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Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) Publication number:

0 190 740 B1

(12)

EUROPEAN PATENT SPECIFICATION

- (45) Date of publication of patent specification: 27.11.91 (51) Int. Cl.⁵: **G01N 33/53, G01N 31/22, G01N 33/52, C12Q 1/04**
- (21) Application number: **86101464.5**
- (22) Date of filing: **05.02.86**

(54) Use of substituted quinone electron transfer agents in analytical determinations.

(30) Priority: **07.02.85 US 699374**

(43) Date of publication of application:
13.08.86 Bulletin 86/33

(45) Publication of the grant of the patent:
27.11.91 Bulletin 91/48

(84) Designated Contracting States:
CH DE FR GB LI SE

(56) References cited:

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EP-A- 113 103	EP-A- 114 267
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Description

This invention relates to clinical chemistry. In particular, it relates to analytical compositions, elements and methods which utilize certain substituted quinone electron transfer agents to determine analytes (e.g. living cells) in aqueous liquids (e.g. biological fluids).

Chemical analysis of liquids, such as water, milk and biological fluids is often desirable or necessary for health maintenance and diagnostic treatment. Various compositions and elements to facilitate such analyses are known. Such compositions and elements generally include a reagent composition for determining a substance under analysis, termed an analyte herein. The analyte can be a biological organism or a nonliving chemical substance. This reagent composition, upon interaction with the analyte, provides a detectable change (e.g. dye formation).

Recently, much work has been directed to developing compositions and elements which are useful for rapid and highly quantitative diagnostic or clinical analysis of biological fluids such as whole blood, serum, plasma, urine and the like.

For the rapid and effective diagnosis and treatment of infectious diseases, it is desirable to be able to detect the bacteria causing the disease as rapidly as possible. Infections of the urinary tract are among the most common bacterial diseases, second in frequency only to infections of the respiratory tract. In fact, in many hospitals, urinary tract infections are the most common form of nosocomial infections, often following the use of catheters and various surgical procedures. Most urinary tract infections (UTI) result from ascending infection by microorganisms introduced through the urethra and vary in severity from an unsuspected infection to a condition of severe systemic disease. Such infections are usually associated with bacterial counts of 100,000 (10^5) or more organisms per ml of urine, a condition referred to as significant bacteriuria. Under normal conditions, urine is sterile, although contamination from the external genitalia may contribute up to 1,000 (10^3) organisms per ml in properly collected and transported specimens.

Significant bacteriuria may be present in a number of pathological conditions involving microbial invasion of any of the tissues of the urinary tract, or may result from simple bacterial multiplication in the urine without tissue invasion. The infection may involve a single site such as the urethra, prostate, bladder, or kidney, although frequently it involves more than one site. Infection restricted to the urine may present itself as asymptomatic bacteriuria, i.e., a condition which manifests no overt signs or symptoms of infection. Early treatment of this condition can prevent the development of more serious conditions, e.g., pyelonephritis (inflammation of the kidney and the renal pelvis). The rapid detection of bacteria by a reliable method would therefore facilitate an early and specific diagnosis.

Further, in order to insure that a prescribed antibiotic is in fact effective in treating an infection, repeated tests during therapy are required. The need for simple, rapid bacteriuria tests is thus clear. Moreover, in view of the frequent unsuspected asymptomatic occurrences of UTI among children, pregnant women, diabetics and geriatric populations, diagnosis of which may require collection and testing of several specimens, bacteriuria tests must be sufficiently simple and economical to permit routine performance. Again, this illustrates the need for a rapid and inexpensive bacteriuria detection method.

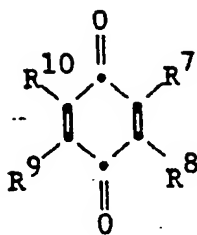
EP-A-0 105 443 (corresponding to US-A-4,629,696) describes the use of certain substituted benzoquinones in an aqueous composition having a pH of 6-9 as redox partners in processes for determination of superoxide dismutase.

Determination of living cells (such as bacteria, yeast, etc.) is best accomplished using a reducible compound which releases a detectable species in the presence of an electron transfer agent. The electron transfer agent is first reduced by the living cell. The reduced electron transfer agent then reduces the reducible compound whereupon the shiftable detectable species is released. Phenazine methosulfate is a known electron transfer agent used in the determination of microorganisms as described in EP-A-0 113 103.

However, phenazine methosulfate and structurally related electron transfer agents have limited stability in aqueous solutions. This instability leads to premature release of the detectable species. This undesirable release is evidenced by high background levels which must be subtracted from assay results to accurately determine the analyte. The background may significantly affect the results when low level analytes (e.g. low levels of bacteria) are being measured.

The problem of premature dye release encountered in the assays described above is solved with an aqueous composition for determination of an analyte which comprises:

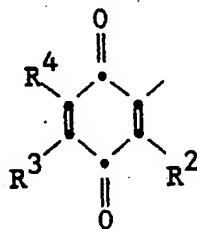
- (a) an electron transfer agent which is capable of being reduced by the analyte,
 - (b) a reducible compound which provides a detectable species when reduced by the reduced electron transfer agent, and
 - (c) a buffer which maintains the composition pH at 9 or less,
- the composition characterized wherein the electron transfer agent has the structure:



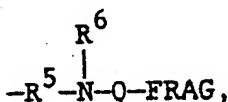
wherein R^7 and R^8 are independently hydrogen, alkyl having 1 to 10 carbon atoms, alkenyl having 1 to 10 carbon atoms, alkoxy having 1 to 10 carbon atoms, hydroxyalkyl having 1 to 10 carbon atoms, hydroxyalkoxy having 1 to 10 carbon atoms, alkoxyalkyl having 2 to 10 carbon atoms, alkoxyalkoxy having 2 to 10 carbon atoms, acetoxyalkyl having 1 to 10 carbon atoms in the alkyl portion, acetoxyalkoxy having 1 to 10 carbon atoms in the alkoxy portion, aryl having 6 to 12 carbon atoms, alkaryl having 7 to 10 carbon atoms, a heterocycle having 5 to 12 carbon, nitrogen, oxygen or sulfur atoms in the ring or a heteroalkyl having 5 to 12 carbon, nitrogen, oxygen or sulfur atoms in the ring, and

R^9 and R^{10} are independently R^7 or R^8 , or taken together supply the atoms to complete a 4- to 8-membered fused carbocyclic or heterocyclic ring with the quinone nucleus, provided at least one of R^7 , R^8 and R^9 and R^{10} is not hydrogen and the electron transfer agent has an $E_{1/2}$ of from -320 to +400 mV as measured in an aqueous solution at pH 7, and

the reducible compound is a dichloroindophenol dye or a compound of the structure CAR- R^1 wherein CAR- is



R^1 is



R^2 and R^4 are independently hydrogen, alkyl having 1 to 40 carbon atoms, aryl having 6 to 40 carbon atoms or an electron withdrawing group having a Hammett sigma value greater than 0.06,

R^3 is R^1 , hydrogen, alkyl having 1 to 40 carbon atoms, aryl having 6 to 40 atoms, or an electron withdrawing group having a Hammett sigma value greater than 0.06, or R^3 and R^4 , taken together, represent the carbon atoms necessary to complete a fused carbocyclic ring with the quinone nucleus, the fused ring having 4 to 8 carbon atoms,

R^5 is alkylene of 1 or 2 carbon atoms,

R^6 is alkyl having 1 to 40 carbon atoms, cycloalkyl having 4 to 40 carbon atoms, heterocycle having 5 to 40 carbon and heteroatoms or aryl having 6 to 40 carbon atoms, provided that when FRAG is a fluorogen, R^6 is methyl,

Q is carbonyl or thiocarbonyl, and

FRAG is a shiftable detectable species which provides a detectable species when released from the reducible compound,

provided that when R^1 is replaced with H, CAR-H has an $E_{1/2}$ of either from +100 to +400 mV when measured in water, or from -650 to -300 mV when measured in acetonitrile, and

further provided that when the reducible compound is reduced at pH 7, at least 50% of FRAG is

released within 30 minutes.

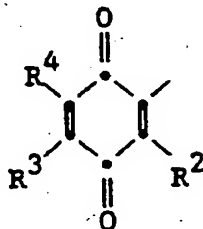
This invention also provides a nonphotosensitive, dry analytical element for determination of an analyte comprising an absorbent carrier material,

the element characterized as containing:

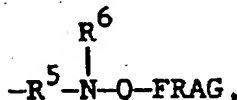
(a) the electron transfer agent described above, and

(b) a reducible compound which provides a detectable species when reduced by the reduced electron transfer agent,

the reducible compound being a dichloroindophenol dye or a compound of the structure CAR-R¹ wherein CAR- is



R¹ is



R² and R⁴ are independently hydrogen, alkyl having 1 to 40 carbon atoms, aryl having 6 to 40 carbon atoms or an electron withdrawing group having a Hammett sigma value greater than 0.06,

R³ is R¹, hydrogen, alkyl having 1 to 40 carbon atoms, aryl having 6 to 40 atoms, or an electron withdrawing group having a Hammett sigma value greater than 0.06, or R³ and R⁴, taken together, represent the carbon atoms necessary to complete a fused carbocyclic ring with the quinone nucleus, the fused ring having 4 to 8 carbon atoms,

R⁵ is alkylene of 1 or 2 carbon atoms,

R⁶ is alkyl having 1 to 40 carbon atoms, cycloalkyl having 4 to 40 carbon atoms, heterocycle having 5 to 40 carbon and heteroatoms or aryl having 6 to 40 carbon atoms, provided that when FRAG is a fluorogen, R⁶ is methyl,

Q is carbonyl or thiocarbonyl, and

FRAG is a shiftable detectable species which provides a detectable species when released from the reducible compound,

provided that when R¹ is replaced with H, CAR-H has an E_{1/2} of either from +100 to +400 mV when measured in water, or from -650 to -300 mV when measured in acetonitrile, and

further provided that when the reducible compound is reduced at pH 7, at least 50% of FRAG is released within 30 minutes.

Further, this invention provides a method for the determination of an analyte in a liquid comprising the steps of:

A. at a pH of 9 or less, contacting a sample of the liquid with

(a) an electron transfer agent capable of being reduced by an analyte as described above, and

(b) a reducible compound which provides a detectable species upon reduction by the reduced electron transfer agent as described above, and

B. detecting the detectable species in the liquid sample as an indication of the presence of the analyte.

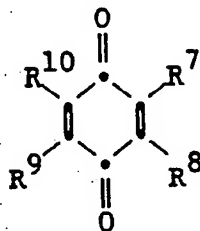
In particular, this invention avoids the problem of premature release of detectable species (e.g. dye) associated with the electron transfer agents taught in the art. The present invention also provides a means for obtaining more rapid and highly sensitive assays, particularly of microorganisms.

The invention provide these unexpected benefits because of the use of an electron transfer agent chosen from a certain class of substituted quinones described in more detail below.

The electron transfer agents useful in this invention are highly compatible with both aqueous and

oleophilic environments. They have sufficient hydrophilic character to be soluble in aqueous buffer solutions. At the same time, they have sufficient oleophilic character to allow interaction with electron donor within the cells.

In general, the electron transfer agents are those having the structure (II):



In this structure, R⁷ and R⁸ are independently hydrogen, substituted or unsubstituted alkyl or alkenyl (generally of 1 to 10 carbon atoms, e.g. methyl, ethyl, chloromethyl, 2-propenyl, n-hexyl, decyl, etc. and preferably of 1 to 5 carbon atoms), substituted or unsubstituted alkoxy (defined similarly to alkyl), substituted or unsubstituted hydroxyalkyl (generally of 1 to 10 carbon atoms, e.g. hydroxymethyl, hydroxyethyl, 2-hydroxypropyl, 2-hydroxyisopropyl, etc. wherein the alkyl portion can be further substituted and preferably of 1 to 5 carbon atoms), substituted or unsubstituted hydroxyalkoxy (defined similarly to hydroxyalkyl), substituted or unsubstituted acetoxyalkyl (generally of 1 to 10 carbon atoms in the alkyl portion of the molecule which can be defined as for alkyl above, e.g. acetoxymethyl, acetoxylethyl, etc. and preferably of 1 to 5 carbon atoms), substituted or unsubstituted acetoxyalkoxy (generally of 1 to 10 carbon atoms in the alkoxy portion of the molecule and defined similarly to acetoxyalkyl above, and preferably of 1 to 5 carbon atoms), substituted or unsubstituted alkoxyalkyl or alkoxyalkoxy (each generally having 2 to 10 carbon atoms with the alkoxy and alkyl portions of the molecule defined similarly to alkyl and alkoxy above), substituted or unsubstituted aryl (generally of 6 to 12 carbon atoms, e.g. phenyl, naphthyl, xylyl, methyl-naphthyl, p-methoxyphenyl, etc.), substituted or unsubstituted alkaryl (generally of 7 to 10 carbon atoms, with the alkyl and aryl portions of the molecule defined similarly to alkyl and aryl above, e.g. benzyl, phenylethyl, p-methoxyphenylethyl, etc.), heterocycle or alkylheterocyclic groups (generally of 5 to 12 carbon, nitrogen, oxygen or sulfur atoms in the ring, with one or more substituents if desired, e.g. morpholino, piperidino, methylpiperidino, etc.). Preferably, R⁷ and R⁸ are independently hydrogen, alkyl, alkoxy or hydroxyalkyl as defined above.

Also, in structure II above, R⁹ and R¹⁰ are independently R⁷ and R⁸, or taken together, supply the carbon, nitrogen, oxygen or sulfur atoms to complete a 4- to 8-membered fused substituted or unsubstituted carbocyclic or heterocyclic ring attached to the quinone nucleus (e.g. to complete a cyclopentane, dihydrofuran, or bicyclic ring, such as bicyclo[2.2.2]octane, benzo, or a bicyclo[2.2.1]heptane ring). Preferably, R⁹ and R¹⁰ are independently one of the groups defined for R⁷ and R⁸, or taken together, supply the carbon atoms to complete a 6- to 8-membered fused carbocyclic ring.

At least one of the substituents, R⁷, R⁸, R⁹ and R¹⁰, is not hydrogen, but is one of the groups defined above, or is taken with another substituent to form the defined fused ring.

It is essential that the electron transfer agents used in the practice of this invention have a reduction potential (E_{1/2}) within the range of from -320 to +400 mV when measured in an aqueous solution at pH 7. Preferably, the E_{1/2} of the ETA is in the range of from -185 to +400 mV. The desired E_{1/2} is achieved by having the appropriate substituents on the quinone nucleus of the compound. With the teaching provided herein, a person skilled in synthetic chemistry would know what substituents to put on the quinone nucleus to obtain the desired E_{1/2}. Reduction potential measurements can be made according to standard electrochemical techniques using either differential pulse polarography or cyclic voltammetry (see, e.g. Sawyer and Roberts, Jr., Experimental Electrochemistry for Chemists, John Wiley & Sons, New York, 1974).

Representative electron transfer agents (ETA) useful in this invention are listed in Table I below in reference to structure II shown above.

Table I

ETA					
Compound	R^7	R^8	R^9	R^{10}	
I	$-\text{CH}_2\text{OH}$	$-\text{H}$	$-\text{CH}_3$	$-\text{CH}_3$	
II	$-\text{OCH}_3$	$-\text{H}$	$-\text{OCH}_3$	$-\text{H}$	
III	$-\text{CH}_3$	$-\text{H}$	$-\text{CH}_3$	$-\text{CH}_3$	
IV	$-\text{OCH}_3$	$-\text{OCH}_3$	$-\text{H}$	$-\text{CH}_3$	
V	$-\text{OCH}_3$	$-\text{H}$	$-\text{H}$	$-\text{OCH}_3$	
VI	$-\text{OCH}_3$	$-\text{OCH}_3$	$-\text{OCH}_3$	$-\text{OCH}_3$	
VII	$-\text{OCH}_2\text{CH}_3$	$-\text{H}$	$-\text{OCH}_2\text{CH}_3$	$-\text{H}$	
VIII	$-\text{CH}_3$	$-\text{CH}_3$	$-\text{CH}_3$	$-\text{CH}_3$	
IX	$-\text{CH}_3$	$-\text{CH}_3$	$-\text{H}$	$-\text{H}$	
X	$-\text{O}(\text{CH}_2)_2\text{OCH}_3$	$-\text{H}$	$-\text{O}(\text{CH}_2)_2\text{OCH}_3$	$-\text{H}$	
XI	$-\text{OCH}_3$	$-\text{OCH}_3$	$-\text{OCH}_3$	$-\text{CH}_3$	
XII	$\begin{array}{c} \text{OH} \\ \\ -\text{CHCH}_3 \end{array}$	$-\text{H}$	$-\text{CH}_3$	$-\text{H}$	
XIII	$-\text{OCH}_3$	$-\text{OCH}_3$	$-\text{H}$	$-\text{H}$	
XIV	$\begin{array}{c} \text{OCH}_3 \\ \\ -\text{CHCH}_3 \end{array}$	$-\text{H}$	$-\text{CH}_3$	$-\text{H}$	

Table I (cont.)





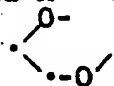







ETA Compound	<u>R⁷</u>	<u>R⁸</u>	<u>R⁹</u>	<u>R¹⁰</u>
5 XV	OH -CHCH ₂ CH ₃	-H	-CH ₃	-H
10 XVI	O OCCH ₃ -CHCH ₃	-H	-CH ₃	-CH ₃
15 XVII	-CH ₃	-H	R ⁹ and R ¹⁰ together form 	
20 XVIII	-OCH ₃	-H	R ⁹ and R ¹⁰ together form 	
25 XIX	-OCH ₃	-H	R ⁹ and R ¹⁰ together form 	
30 XX	-OCH ₃	-H	R ⁹ and R ¹⁰ together form 	
35 XXI	-CH ₃	-H	R ⁹ and R ¹⁰ together form 	
40 XXII	-CH ₂ CH ₂ OH	-H	-OCH ₃	-H

Table I (cont.)

ETA	Compound	R ⁷	R ⁸	R ⁹	R ¹⁰
5	XXIII	-OCH ₃	-OCH ₃	R ⁹ and R ¹⁰ together form	
10	XXIV	-OCH ₃	-H	R ⁹ and R ¹⁰ together form	
15	XXV	-CH ₃	-H	R ⁹ and R ¹⁰ together form	
20	XXVI	-CH ₂ CH ₂ OH	-H	R ⁹ and R ¹⁰ together form	
25	XXVII	-CH ₂ OH	-H	R ⁹ and R ¹⁰ together form	
30	XXVIII	-CH ₃	-CH ₃	R ⁹ and R ¹⁰ together form	
35	XXIX	-CH ₂ OH	-CH ₃	R ⁹ and R ¹⁰ together form	

ETAs I, III, IV, XXVI and XXVII of Table I are particularly useful in the practice of this invention, with ETAs I, III and IV (2,3-dimethyl-5-hydroxymethyl-1,4-benzoquinone and 2,3,5-trimethyl-1,4-benzoquinone and 2,3-dimethoxy-5-methyl-1,4-benzoquinone, respectively) being most preferred.

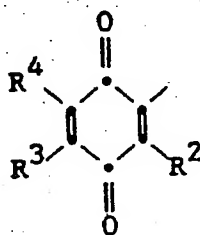
The electron transfer agents described herein can be prepared using starting materials and procedures known in the art to a skilled synthetic chemist. Generally, they are prepared according to the following sequence of reactions: (1) protection of the hydroquinone groups, (2) attachment of the appropriate substituents to the protected hydroquinone nucleus, (3) removal of the protecting groups, and (4) conversion of the hydroquinone to a quinone. Representative preparations of some electron transfer agents are provided in the illustrative preparations preceding the Examples below.

The electron transfer agents are used in combination with a reducible compound which can provide a detectable species when reduced by the agent. The detectable species can be obtained by the reducible compound undergoing a change to become detectable. Alternatively, the detectable species can be

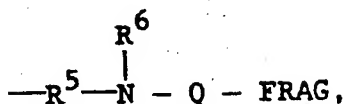
obtained by release from the reducible compound. The detectable species can be a material which is directly detectable by a suitable means, as well as a material which can react with other substances, e.g. other analytes, enzymes, mordants, metal ions or other materials to provide a detectable species. Such species includes those detectable by radiometric means, including chromogens (e.g. dyes or pigments) which can be detected colorimetrically, and fluorogens (e.g. fluorescent dyes or probes) which can be detected fluorometrically. Additionally, the detectable species can be a phosphorescent species, a radioactively tagged species, or a chemiluminescent species, or any other detectable species known to one skilled in the art.

Useful reducible compounds include dichloroindophenol dyes which can be reduced to colorless compounds, and other dye-providing materials which can be reduced.

Preferred reducible compounds are represented by the structure CAR-R' wherein CAR- is



R' is



wherein R⁵ is substituted or unsubstituted alkylene, preferably of 1 or 2 carbon atoms in the backbone (e.g. methylene, ethylene, alkoxyethylene, etc.). Most preferably, R⁵ is methylene. Q is carbonyl or thiocarbonyl and preferably carbonyl.

R⁶ is substituted or unsubstituted alkyl preferably of 1 to 40 carbon atoms (e.g. methyl, ethyl, n-propyl, isopropyl, t-butyl, hexyl, decyl, lauryl, benzyl, etc.), substituted or unsubstituted cycloalkyl preferably of 4 to 40 carbon atoms (e.g. cyclobutyl, cyclohexyl, 4-methylcyclohexyl, etc.), or substituted or unsubstituted aryl of 6 to 40 carbon atoms (e.g. phenyl, xylyl, naphthyl, p-nitrophenyl, anthryl, p-t-butoxyphenyl, etc.). Preferably, R⁶ is lower alkyl of 1 to 3 carbon atoms (substituted or unsubstituted), and more preferably, R⁶ is methyl.

FRAG is a shiftable detectable species which, when cleaved from the RIND compound, provides a detectable species. This species is released in an amount which can be directly related to the amount of reductant present. The specific composition of FRAG can vary considerably depending upon the type of detectable species desired and upon the particular detection means employed.

The detectable species can be a material which is directly detectable by a suitable means, as well as a material which can react with other substances, e.g. other analytes, enzymes or other reagents to provide a detectable species. Such species include chromogens (e.g. dyes or pigments) which can be detected colorimetrically and fluorogens (e.g. fluorescent dyes or probes) which can be detected fluorometrically. Additionally, the detectable species can be a phosphorescent species, or a chemiluminescent species, or any other detectable species known to one skilled in the art.

Particularly useful shiftable detectable moieties are chromogens and fluorogens having a first spectral absorption band prior to release and a second spectral absorption band when measured after release. Examples of useful classes of chromogens are azo, azomethine, nitrophenol, indophenol, indoaniline and triarylmethane dyes, and others known in the art, with azo dyes being preferred. Examples of useful classes of fluorogens are coumarin, fluorescein and rhodamine fluorescent dyes, and others known in the art.

Useful phosphorescent species include such phosphors as 2',5'-dibromofluorescein and 4',5'-diodofluorescein. A useful chemiluminescent species is luciferin.

FRAG generally is linked to Q through a bivalent monoatom linkage which is a part of FRAG. Preferably, the monoatom linkage is oxy or thio.

R², R³ and R⁴ in the above quinone structure are independently hydrogen, substituted or unsubstituted alkyl of 1 to 40 carbon atoms (e.g. methyl, ethyl, hydroxymethyl, methoxymethyl, etc.) substituted or unsubstituted aryl (e.g. phenyl, naphthyl, methylnaphthyl, p-nitrophenyl, m-methoxyphenyl, phenylsulfonamido, etc.) or an electron withdrawing group which generally has a positive Hammett sigma value, and preferably has a sigma value greater than about 0.06. Hammett sigma values are calculated in accordance with standard procedures described e.g. in *Steric Effects in Organic Chemistry*, John Wiley & Sons, Inc., 1956, pp. 570-574 and *Progress in Physical Organic Chemistry*, Vol. 2, Interscience Publishers, 1964, pp. 333-339. Typical useful electron withdrawing groups having positive Hammett sigma values include cyano, carboxy, nitro, halo (e.g. fluoro, bromo, chloro, iodo), trihalomethyl (e.g. trifluoromethyl, trichloromethyl, etc.), trialkylammonium, carbonyl, carbamoyl, sulfonyl, sulfamoyl, esters and others known in the art, or alkyl or aryl groups (defined above) substituted with one or more of these electron withdrawing groups. Preferred electron withdrawing groups include nitro, cyano, p-nitrophenyl, p-cyanophenyl and 2,5-dichlorophenyl. Aryl groups with methoxy or acetamido groups in the meta position are also useful.

R³ can also be R¹ thereby potentially providing a 2:1 molar ratio of detectable species to RIND compound.

Alternatively, R³ and R⁴, taken together, can represent the carbon atoms necessary to complete a substituted or unsubstituted fused carbocyclic ring attached to the quinone nucleus. For example, such a ring (mono- or bicyclic) can have from 4 to 8, and preferably from 5 to 7, carbon atoms in the backbone.

It is essential that when R¹ of CAR-R¹ is replaced by a hydrogen atom, CAR-H has an E_{1/2} of either at least +100 mV when measured in water, or of at least -650 mV when measured in acetonitrile. Such measurements are made according to standard electrochemical techniques using either differential pulse polarography or cyclic voltametry (see, e.g. Sawyer & Roberts, Jr., reference, noted above). Preferably, the E_{1/2} is from +100 to +400 mV as measured in water or from -650 to -300 mV as measured in acetonitrile. The desired E_{1/2} is achieved by having appropriate electron withdrawing groups on the CAR- nucleus, or by a combination of a fused ring attached to the nucleus and electron withdrawing groups.

The electron transfer agent and reducible compound can be combined with a buffer. Useful buffers include those which will maintain the pH of the composition at 9 or less, and preferably from 6.5 to 8. Representative buffers include phosphates, borates, N-2-hydroxy-ethylpiperazine-N'-2-ethane sulfonic acid, and other buffers known in the art, e.g. those described by Good et al in *Biochem.*, 5, p. 467 (1966) and *Anal. Biochem.*, 104, 300 (1980).

The compositions of this invention are useful for analytical determination (i.e. quantitative or qualitative detection) of aqueous or non-aqueous liquids, e.g. biological fluids, manufacturing processes, wastewater, food stuffs, etc. Determinations can be made of various analytes, including living cells (e.g. bacteria, yeast, fungi, etc.), enzymes (e.g. lipase, glucose oxidase, lactate oxidase, creatine kinase, α -glycerophosphate oxidase, lactate dehydrogenase, alanine aminotransferase, aspartate aminotransferase and other NADH-based, FADH-based or peroxidase-based assays which include dehydrogenase or reductase enzymes), biological or chemical reductants other than living cells which will reduce the electron transfer agent (e.g. ascorbate, cysteine, glutathione, etc.), metabolizable substances (e.g. glucose, lactic acid, triglycerides, cholesterol, etc.), immunoreactants (e.g. antigens, antibodies, haptens, etc.), and other determinations made via a single reaction or sequence of reactions which brings about reduction of the reducible compound and release of a detectable species.

The compositions of this invention are particularly useful in detecting or quantifying living cells in biological samples. Although any biological sample suspected of having living cells therein (e.g. food, tissue, ground water, cooling water, pharmaceutical products, sewage, etc.) can be analyzed for bacteria, yeast, fungi, etc. by this invention, the invention is particularly useful for bacterial detection in aqueous liquids, such as human and animal fluids (e.g. urine, cerebral spinal fluid, blood and the like as well as stool secretions) and suspensions of human or animal tissue. The practice of this invention is particularly important for detection of urinary tract infections in urine (diluted or undiluted).

The detection of living cells, and particularly of bacterial cells, is often carried out in the presence of a nutrient for those cells although its presence is not essential. Any nutrient media can be used which contains useful carbon, and optionally nitrogen, sources. Suitable nutrient media having proper components and pH are known in the art.

The present invention is adaptable to either solution or dry assays. For solution assay, the composition of this invention is contacted with liquid test sample containing the living cells or analyte to be determined and mixed. Alternatively, the components of the composition can be separately added to the test sample. Generally the analytical composition is mixed with the test sample in a suitable container (e.g. test tube, petrie dish, beaker, cuvette, etc.). The resulting solution (or dispersion) is incubated for a relatively short time (i.e. up to about 30 minutes) at a temperature up to about 40° C, and generally from about 20 to about

40° C. The test sample is then evaluated by measuring the detectable species (e.g. chromogen or fluorogen) that has been released by reduction of the reducible compound. Such an evaluation can be done with suitable detection equipment, generally 60 minutes after the contacting of the reagents and test sample.

- 5 The solution assay can also be carried out by contacting a porous, absorbent material, e.g. paper strip, containing the test sample with the analytical composition. The analyte in the test sample can migrate from the porous material into the composition thereby initiating the analytical reactions needed for the determination.

- 10 Generally, in a solution assay, the amount of reducible compound present is from 0.01 to 10, and preferably from 0.05 to 1, millimolar. The electron transfer agent is generally present in an amount of from 0.01 to 2, and preferably from 0.05 to 1, millimolar. Other reagents can be present in amounts readily determined by one skilled in the art.

- Alternatively, this invention can be practiced in a "dry" assay which utilizes a dry analytical element. Such an element can be a simple absorbent carrier material, i.e. a thin sheet or strip of self-supporting
15 absorbent or bibulous material, such as filter paper or strips, which contains the electron transfer agent, and optionally the reducible compound, or a dried residue of same. Such elements are known in the art as test strips, diagnostic elements, dip sticks, diagnostic agents and the like.

- When employed in dry analytical elements, the reagents can be incorporated into a suitable absorbent carrier material by imbibition or impregnation, or can be coated on a suitable absorbent material. Useful
20 carrier materials are insoluble and maintain their structural integrity when exposed to water or physiological fluids such as urine or serum. Useful carrier materials can be prepared from paper, porous particulate structures, cellulose, porous polymeric films, wood, glass fiber, woven and nonwoven fabrics (synthetic and nonsynthetic) and the like. Useful materials and procedures for making such elements are well known in the art as exemplified by US-A-3,092,465, US-A-3,802,842, US-A-3,915,647, US-A-3,917,453, US-A-3,936,357,
25 US-A-4,248,829, US-A-4,255,384, and US-A-4,270,920, and GB-A-2,052,057.

- A dry assay can be practiced to particular advantage with an analytical element comprising a support having thereon at least one porous spreading zone as the absorbent carrier material. The reagents can be in the spreading zone or in a different zone (e.g. reagent zone, registration zone, hydrophilic zone, etc.) or in separate zones. The spreading zone can be prepared from any suitable fibrous or non-fibrous material or
30 mixtures of either or both. The void volume and average pore size of this zone can be varied depending upon the use intended. For example, if whole blood or other liquid samples containing cells or high molecular weight materials are to be assayed, the void volume and average pore size are generally greater than if serum or urine is to be assayed.

- Useful spreading zones can be prepared using as described in US-A-4,292,272, US-A-3,992,158, US-A-
35 4,258,001 and US-A-4,430,436 and JP-57(1982)-101760.

- The dry analytical elements of this invention can be a single self-supporting porous spreading zone containing the desired reagents for a particular assay, but preferably such zone is carried on a suitable nonporous support. Such a support can be any suitable dimensionally stable, and preferably, transparent (i.e. radiation transmissive) film or sheet material which transmits electromagnetic radiation of a wavelength
40 between 200 and 900 nm. A support of choice for a particular element should be compatible with the intended mode of detection (reflection, fluorescence or transmission spectroscopy) and inert to chemical reagents and liquid samples used in the assay. Useful support materials include polystyrene, polyesters [e.g. poly(ethylene terephthalate)], polycarbonates, cellulose esters (e.g. cellulose acetate), etc.

- The elements can have more than one zone, e.g. a reagent zone, a registration zone, radiation-blocking
45 zone, subbing zone, etc. The zones are generally in fluid contact with each other, meaning that fluids, reagents and reaction products can pass between superposed regions of adjacent zones. Preferably, the zones are separately coated superposed layers, although two or more zones can be in a single layer. Besides the references noted above, suitable element formats and components are described, for example, in US-A-4,042,335 and US-A-4,144,306 and US Reissue 30,267.

- 50 In the elements of this invention, the amount of the reducible compound can be varied widely, but it is generally present in a coverage of up to 1, and preferably from 0.05 to 0.2, g/m². The electron transfer agent is generally present in a coverage of up to 10, and preferably from 0.01 to 1, g/m². Optional, but preferred reagents (e.g. nutrient, buffer, etc.) are generally present in the following coverages:

- | | | |
|----|------------------|---|
| 55 | nutrient: | generally up to 10, and preferably from 0.1 to 2, g/m ² , |
| | buffer (pH ≤ 9): | generally up to 5, and preferably from 0.5 to 2, g/m ² , and |
| | surfactant: | generally up to 10, and preferably from 0.2 to 5, g/m ² . |

One or more of the zones can contain a variety of other desirable, but optional, components, including activators, binders, coupler solvents, etc. as is known in the art, as well as any reagents needed for assay of

a particular analyte.

In one embodiment of this invention, an element for detection of microorganisms (e.g. yeast, fungi, bacteria, etc.) comprises an absorbent spreading zone containing an electron transfer agent and a RIND compound, both of which are described above. It is desirable that these elements also contain a nutrient for the living cells and a buffer which maintains physiological pH under conditions of use (i.e. when contacted with a 1-100 μ l sample of liquid). Such an element can be used to detect bacteria, for example, in a urine sample (generally pretreated to eliminate reductive interferents) by physically contacting the sample and element in a suitable manner, and detecting any moiety released from the RIND compound when it is reduced.

In another embodiment of this invention, an element for the determination of a nonliving biological or chemical analyte in an aqueous liquid comprises an interactive composition which is capable of providing a detectable species upon interaction with the analyte. This composition comprises a reducible compound which releases the detectable species when reduced, an electron transfer agent, and optionally, a nonionic surfactant and a buffer which maintains physiological pH during the assay, all of which are described above. Examples of such analytes are described above. The elements contain interactive compositions having suitable reagents which effect reduction of the electron transfer agent and reducible compound and release of a detectable species. The amount of detectable species detected can be correlated to the amount of analyte present in the liquid sample.

A variety of different elements, depending on the method of assay, can be prepared in accordance with the present invention. Elements can be configured in a variety of forms, including elongated tapes of any desired width, sheets, slides or chips.

The assay of this invention can be manual or automated. In general, in using the dry elements, an analyte or living cell determination is made by taking the element from a supply roll, chip packet or other source and physically contacting it with a sample (e.g. 1-100 μ l) of the liquid to be tested so that the liquid mixes with the reagents within the element. Such contact can be accomplished in any suitable manner, e.g. dipping or immersing the element into the sample or, preferably, by spotting the element by hand or machine with a drop of the sample with a suitable dispensing means.

After sample application, the element is exposed to any conditioning, such as incubation, heating or the like, that may be desirable to quicken or otherwise facilitate obtaining any test result.

Detection of an analyte or living cell is achieved when the reducible compound is reduced releasing a species which can be detected in a suitable manner. Generally the detection is carried out within 60 minutes from the time of initial contact of reagents with the test sample, whether in a dry or solution assay. Preferably, as noted above, the detectable species is a colorimetric dye or fluorescent dye which can be detected with standard colorimetric or fluorometric apparatus and detection procedures. If the detectable species is other than a chromogen or fluorogen, for example, a radioisotope, chemiluminescent or phosphorescent moiety, standard radioisotopic, chemiluminescence or phosphorescence detecting means can be employed.

For solution assays described below, emulsions of the RIND compounds were prepared by dissolving the RIND compound in N,N-dimethylformamide (DMF), adding surfactant, and then adding this solution to aqueous potassium phosphate (KP) buffer.

For examples 3-6, cells were grown in brain heart infusion medium at 37°C and transferred daily. Cultures of *Pseudomonas aeruginosa* (ATCC 27853) were grown with shaking. All other organisms were grown in static culture. Forty ml of cells that were grown overnight were harvested, washed and resuspended in 10 ml of 0.05 molar KP buffer (pH 7.5). A stock solution was prepared with an approximate cell concentration of 3×10^7 cells/ml as determined by reading the optical density at 620 nm in a spectrophotometer. An optical density of 0.05 units corresponds to an approximate cell density of 3×10^7 cells/ml.

Measurement of dye release was determined in the following manner. The reaction mixture to be tested contained the following: the appropriate electron transfer agent (ETA) solution (phenazine methosulfate, phenazine ethosulfate, 3 mg/ml in methanol, ETAs of this invention, 1.5 mg/ml in methanol), glucose and tryptose as cell nutrients, KP buffer (pH 7.5) and an aliquot of the microemulsion. After equilibration at 37°C, the reaction was initiated by the addition of an aliquot of the microorganism, typically 10-50 μ l of a 10^8 cells/ml suspension. Spectrophotometric absorbance was determined in a commercially available Perkin Elmer spectrophotometer at 636 nm. The results are usually expressed as net signal, i.e., the density produced by the sample minus the density for the control which contained all the reactants except the cells.

For examples 1, 2, 7 and 8, solution studies of ETA reactivity and background were carried out as follows:

E. coli cells (ATCC 25922) were grown in brain heart infusion (BHI) medium at 37°C without shaking.

Forty milliliters of cells that were grown overnight were harvested by centrifugation. The pellet was resuspended in 25 ml of buffer. An aliquot was diluted with the same buffer to obtain an optical density of 0.1 at 620 nm, as read by a commercially available Cary 219 spectrophotometer, and measured against a buffer blank. An optical density of 0.1 at 620 nm has been determined to correspond to a cell concentration of 6×10^7 cells/ml. A stock solution was prepared with a cell concentration of 6×10^9 cells/ml.

An aqueous composition of a RIND compound in DMF, surfactant and buffer (pH 7.5) was treated with 10% glucose solution and an aliquot of the cell stock solution. After equilibration at 37°C, the reaction was initiated by the addition of the appropriate ETA, dissolved in methanol. Reactions were followed by monitoring the appearance of dye at 636 nm using a Cary 219 spectrophotometer. For each ETA, experiments were performed on different days using freshly prepared cells. Multiple determinations of cellular dye release and background dye release (RIND compound and ETA) were obtained.

Reduction potentials (measured as the half-wave potentials $E_{1/2}$) were determined using the differential pulse polarographic technique. A commercially available PAR Model 174 polarographic analyzer was used. The solution medium was sodium phosphate buffer (pH 7.0, μ -0.1). Measurements were made against a standard calomel electrode. Values are reported versus the normal hydrogen electrode. The reduction potentials of the ETAs are listed in Table II.

The following preparations are representative of procedures used to prepare ETAs useful in the practice of this invention. The intermediate and final compounds were characterized by mass spectral, nuclear magnetic resonance (NMR) and elemental analyses.

Preparation 1- Preparation of 2,3-Dimethyl-5-hydroxymethyl-1,4-benzoquinone (ETA I)

A mixture of 2,3-dimethylbenzoquinone (47.6 g, 0.35 mole) and 10% palladium on carbon catalyst in 100 ml tetrahydrofuran was shaken on a Paar apparatus under 40 psi (2.75 bars) of hydrogen for 1 hour at room temperature. The reaction mixture was filtered under a nitrogen atmosphere and the filtrate concentrated under vacuum to yield 39.9 g of 2,3-dimethyl-1,4-hydroquinone (intermediate A).

Methyl iodide (71.6 ml, 1.15 mole) was added to a stirring mixture of intermediate A (39.9 g, 0.288 mole), finely ground potassium carbonate (119.7 g, 0.866 mole) and dry acetone (230 ml). This mixture was refluxed for four days under a nitrogen atmosphere. The reaction mixture was filtered hot and the filtrate was concentrated to remove most of the acetone. The residue was added to 10% hydrochloric acid in ice water, and the precipitated solid was collected and dried. The yield was 45.2 g of 2,3-dimethyl-1,4-dimethoxybenzene (intermediate B).

Hydrogen chloride gas was bubbled into a stirred mixture of formaldehyde (24 ml, 37% in water), dioxane (48 ml) and concentrated hydrochloric acid (12 ml) for 15 minutes. The reaction was exothermic up to 75°C. When the temperature had dropped to 65°C, intermediate B (20 g, 0.12 mole) in 60 ml of dioxane was added dropwise to the mixture over 15 minutes. Hydrogen chloride gas was again bubbled into the reaction mixture for 10 minutes, after which the solution was stirred for 1 hour at room temperature. The solution was then poured into 1 liter of ice water, the precipitated material was collected and dried to yield 25.4 g of 5-chloromethyl-1,4-dimethoxy-2,3-dimethylbenzene (intermediate C).

A mixture of intermediate C (5 g, 0.023 mole) and anhydrous sodium acetate (7.1 g, 0.086 mole) in glacial acetic acid (35 ml) was refluxed for 20 hours. The reaction mixture was cooled to room temperature, diluted with 50 ml of water and extracted with dichloromethane. The organic extracts were washed with saturated sodium bicarbonate solution, dried, and concentrated to yield a dark syrup, which eventually crystallized to give 5.1 g of crude 5-acetoxymethyl-1,4-dimethoxy-2,3-dimethylbenzene (intermediate D).

A solution of intermediate D (5.1 g, 0.21 mole) in 100 ml of methanol and 25 ml of 10% sodium hydroxide was refluxed for one hour, then poured into ice water. The resulting solid was collected by filtration and dried to give 1.8 g of 1,4-dimethoxy-2,3-dimethyl-5-hydroxymethylbenzene (intermediate E).

To a mixture of intermediate E (1.8 g, 9 mmole), sodium acetate (7.5 g, 91 mmole), acetonitrile (134 ml) and water (29 ml), silver dipicolinate complex (19.2 g, 44 mmole, prepared by the method described by K. Kloc et al in *Chem. Lett.*, 725, 1980) was added slowly with vigorous stirring over 30 minutes at room temperature. The mixture was stirred over 30 minutes longer, water (150 ml) was added, and the silver salts were filtered off and washed with chloroform. The filtrate was extracted several times with chloroform. The combined extracts were dried, concentrated to a small volume and chromatographed (silica, dichloromethane:ethyl acetate, 95:5). There was obtained 900 mg of ETA I, mp 71-72°C. Calculated elemental analysis for $C_9H_{10}O_3$ is C, 65.1, H, 6.1, and O, 28.9. Elemental analysis found was C, 64.9, H, 5.9, and O, 28.7.

Preparation 2- Preparation of 5-Methylindane-4,7-dione (ETA XVII)

4,7-Dimethoxy-1-indanone, (intermediate A), prepared by the method of R. T. Coutts et al (Can. J. Chem., 52, 381, 1974), was obtained as yellow needles from ethanol, mp 123-126° C.

A mixture of intermediate A (19 g, 99 mmole), perchloric acid (2.5 ml of a 70% aqueous solution) and 10% palladium on carbon catalyst (2 g) in acetic acid (250 ml) was placed in a Paar bottle and shaken under 40 psi (2.75 bars) of hydrogen in a Paar shaker apparatus for 15 hours. Solid potassium acetate was added, and the mixture was filtered through a standard Celite pad. The pad was washed with tetrahydrofuran, and the combined filtrates were poured into ice water (2 liters). The resulting white solid was isolated by filtration, washed with water, and dried under vacuum. Chromatography (silica, dichloromethane) afforded the desired product, 4,7-dimethoxyindane (intermediate B) as a white solid (8.5 g).
 10 White needles were obtained from ethanol:water (1:1), mp 82-84°.

Hydrogen chloride gas was bubbled into a stirred mixture of formalin (2.2 ml, 37% in water), dioxane (4.4 ml) and concentrated hydrochloric acid (1.1 ml) for 15 minutes. The reaction was exothermic up to 75° C. When the temperature had dropped to 65° C, 4,7-dimethoxyindane (2.0 g, 11 mmole) in 10 ml of dioxane was added dropwise over 10 minutes. Hydrogen chloride gas was again bubbled into the reaction mixture for 10 minutes, after which the solution was stirred for one hour at room temperature. The reaction mixture was then poured into 600 ml ice water. The precipitated material was collected by filtration and dried to yield 2.3 g of 5-chloromethyl-4,7-dimethoxyindane (intermediate C).

A mixture of intermediate C (2.3 g, 10 mmole), ethanol (150 ml) and 10% palladium on carbon catalyst was added to a Paar bottle and shaken under 40 psi (2.75 bars) of hydrogen in a Paar shaker apparatus for 24 hours at room temperature. The reaction mixture was filtered through a standard Celite pad, washed with ethanol, and the filtrate concentrated to yield 2.1 g of 5-methyl-4,7-dimethoxyindane (intermediate D).

Cerrie ammonium nitrate (18.1 g, 33 mmole) in acetonitrile (25 ml) and water (6 ml) was added dropwise to a stirred mixture of intermediate D (2.1 g, 11 mmole) in acetonitrile (25 ml). After one hour at room temperature, the reaction mixture was extracted with chloroform. The organic layer was dried and concentrated under reduced pressure to yield the crude product. This material was purified by column chromatography (silica, 95:5, dichloromethane:ethyl acetate) and rotary chromatography (silica, 80:20, dichloromethane:lignoin). There was obtained 570 mg of ETA XVII, mp 45-47° C. Calculated elemental analysis for C₁₀H₁₀O₂ is C, 74.1, H, 6.2, and O, 19.7. Elemental analysis found was C, 73.7, H, 6.1, and O, 20.1.

30 Preparation 3- The Preparation of 6,7-Dimethoxy-1,2,3,4-tetrahydro-1,4-ethanonaphthalene-5,8-dione (ETA XXIII)

Intermediate A, 2,3-dimethoxybenzoquinone, was prepared by the procedure of J. C. Catlin et al (J. Med. Chem., 14:45, 1971).

A mixture of intermediate A (1 g, 6 mmole), 1,3-cyclohexadiene (0.6 ml, 6 mmole), and toluene (15 ml) was refluxed about 15 hours under a nitrogen atmosphere. The solvent was removed under reduced pressure to yield 1.5 g of crude Diels-Alder adduct (intermediate B), which was used directly in the next step.

Intermediate B (1.5 g, 6 mmole) and potassium bicarbonate (1.23 g, 12 mmole) in 30 ml of dry methanol were refluxed under a nitrogen atmosphere for 30 minutes. The reaction mixture was filtered, and the filtrate was poured into dilute hydrochloric acid and ice water to precipitate the crude product. This crude material was dissolved in tetrahydrofuran (100 ml) and placed in a Paar bottle with 10% palladium on carbon catalyst, and shaken under 40 psi (2.75 bars) hydrogen in a Paar shaker apparatus for 2 hours. The reaction mixture was filtered, and the filtrate was concentrated under reduced pressure to yield 700 mg of a white solid, 6,7-dimethoxy-1,2,3,4-tetrahydro-1,4-ethano-5,8-dihydroxynaphthalene (intermediate C) which was used directly in the next step.

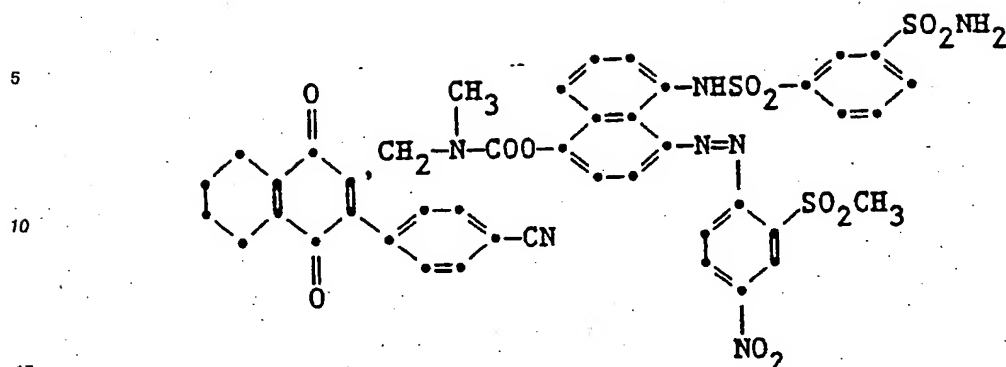
Intermediate C (700 mg) was dissolved in dichloromethane (70 ml) and lead peroxide (2 g) was added. After 3 hours at room temperature, the reaction mixture was filtered, and the filtrate was concentrated to a small volume and chromatographed (silica, dichloromethane:ethyl acetate, 95:5) to yield 600 mg of ETA XXIII, mp 104.5-105.5° C. Calculated elemental analysis for C₁₄H₁₆O₄ is C, 67.7, H, 6.5, and O, 25.8. Elemental analysis found was C, 67.6, H, 6.2, and O, 25.6.

The following examples are provided to illustrate the practice of this invention.

55 Example 1 - Comparative Example Using RIND IX

This example compares the present invention using the ETAs described herein with embodiments using phenazine methosulfate (PMS) and phenazine ethosulfate (PES). The reducible compound (RIND) used in

this comparison had the structure:



The following solutions were prepared:

- (1) 9.8×10^{-3} molar ETA in MeOH for each ETA,
- (2) 1.7×10^{-2} molar RIND compound in N,N-dimethylformamide (DMF),
- (3) 50 μ l of solution 2 + 100 μ l of TRITON X-100 + 5 ml of potassium phosphate (KP) buffer,
- (4) 10% glucose solution, and
- (5) 6×10^9 cells/ml in KP buffer.

Solutions 1, 3 and 5 were prepared daily. A 1 mm pathlength cuvette (400 μ l capacity) was filled with 188 μ l of solution 3, 188 μ l of KP buffer, 6 μ l of solution 4, and 12.5 μ l of solution 5. The cuvette was covered with a rubber serum cap, to prevent evaporation, and thermally equilibrated to 37° C. Once equilibrated, the reaction was initiated by the addition of 3 μ l of solution 1. Final solution concentrations of the initial reactants were: ETA (7.7×10^{-5} molar), RIND compound, (7.6×10^{-5} molar), *E. coli* cells (6×10^7 cells/ml) and glucose (8.8×10^{-3} molar). Reactions were followed by monitoring the appearance of dye at 636 nm using a Cary 219 spectrophotometer.

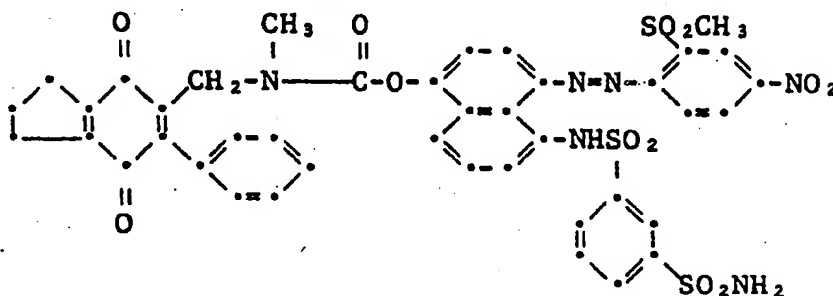
For each ETA, three or four determinations were performed on different days. Multiple determinations of background dye release (RIND compound + ETA) were also obtained. Table II below lists data showing background and reduction potentials ($E_{1/2}$) of the ETAs. All of the ETAs of this example provided lower backgrounds than PMS after 10 minutes, and all but ETA XXVI provided lower background than PES after 10 minutes.

Table II

ETA	% Background Release (10 Min)	% Background Release (30 Min)	E _{1/2} (mV)
PMS	1.7	3.5	96
PES	1.0	2.1	83
I	0.6	1.4	150
II	0.4	2.2	84
III	0.3	1.5	121
IV	0.6	1.4	154
V	0.3	1.5	102
VI	0.3	1.0	124
VII	0.4	1.8	50
VIII	0.1	0.6	60
IX	0.3	1.3	184
X	0.6	2.8	90
XI	0.3	1.1	126
XVII	0.3	0.8	176
XXVI	1.3	2.1	12
XXVII	0.7	1.6	22
XXVIII	0.2	0.8	122

Example 2 - Comparative Example Using RIND XX

Dye release from a RIND compound illustrated below was measured at 636 nm using the ETAs of the present invention and PMS under the same experimental conditions described in Example 1. The RIND compound used had the structure:



The results, listed in Table III below, indicate that all of the ETAs tested provided less background density than PMS, except ETA XXIX, which gives a slightly higher background after 10 minutes and about the same background after 30 minutes. Preferred ETAs I and III provided lower background and very fast dye release.

Table III

ETA	Time (Min) for 100% Dye Release	% Background Release (10 Min)	% Background Release (30 Min)
PMS	20	2.2	4.5
I	18	1.6	2.7
III	18	0.5	1.8
XVII	43	1.1	1.9
XXVI	25	1.5	3.1
XXVII	26	1.4	2.4
XXVIII	34	0.3	1.1
XXIX	29	3.2	4.6

Example 3 -

Comparison of Response of ETAs in Determination of *Pseudomonas* *aeruginosa*

Test solutions were prepared with the following components: 1.5 ml of an aqueous composition of the RIND compound shown in Example 1, 25 μ l of the appropriate ETA solution, 25 μ l of glucose (5% solution) and 1.5 ml of potassium phosphate buffer (pH 7-7.5). After equilibration of the test solutions at 37° C, a 25 μ l aliquot of an urinary tract infection (UTI) microorganism, *P. aeruginosa*, (about 1×10^8 cells/ml) was

added, and the reaction was followed at 635 nm in a commercially available Perkin-Elmer Lambda 5 spectrophotometer. A Control solution without ETA was likewise monitored. Table IV below shows the absorbance change (ΔA) between sample and control after 15 and 30 minutes. The determinations using ETAs I and III show unexpectedly higher responses compared to those using phenazine methosulfate (PMS) and phenazine ethosulfate (PES).

Table IV

	ΔA (15 min)	ΔA (30 min)
ETA I	0.683	1.921
ETA III	0.534	1.758
PMS	0.141	0.394
PES	0.088	0.237
Control	0.092	0.256

Example 4 -

Response Using Tryptose as an Additional Nutrient with *P. aeruginosa*

Test solutions were prepared from the following components: 1.5 ml of an aqueous composition of the RIND compound shown in Example 1, 25 μ l of the appropriate ETA solution, 25 μ l of glucose (10% solution), 25 μ l of tryptose (10% solution), and 1.5 ml of potassium phosphate buffer (pH 7.5). After equilibration of the test solution at 37° C, a 25 μ l aliquot of *P. aeruginosa* (approx. 1×10^8 cells/ml) was added to each to give reaction mixtures, and the reaction was followed at 635 nm as in Example 3. Table V below shows the absorbance change (ΔA) after 15 and 30 minutes for two runs of the reaction mixtures. Both reaction mixtures 1 and 2 show improved dye release over all of the Control mixtures after both 15 and 30 minutes. The addition of tryptose as an additional nutrient gives a higher response.

Table V

Rxn Mixture	ΔA 15 minutes	ΔA 30 minutes
1	1.076/1.061	4.008/3.987
2	0.570/0.430	1.849/1.561
Control A	0.134/0.135	0.393/0.412
Control B	0.030/0.031	0.074/0.089
Control C	0.061/0.058	0.161/0.170

Rxn Mixture 1 = *P. aeruginosa*, RIND compound, ETA III, glucose, tryptose
 Rxn Mixture 2 = *P. aeruginosa*, RIND compound, ETA III, glucose
 Control A = *P. aeruginosa*, RIND compound, PMS, glucose
 Control B = ETA III, glucose, tryptose
 Control C = PMS, glucose

Example 5 -

Comparison of Response of ETAs in Determination of Several Organisms

Test solutions were prepared from the following components: 1.5 ml aqueous composition of the RIND compound shown in Example 1, ETA III and PMS (25 μ l of the ETA solution), 25 μ l of glucose (10% solution), 25 μ l of tryptose (10% solution), and 1.5 ml of potassium phosphate buffer. After equilibration of the test solutions at 37°C, a 25 μ l aliquot of the appropriate organism (approx. 1×10^8 cells/ml) was added and the reaction in each solution was followed at 635 nm as in Example 3. Table VI below shows the superior response of ETA III compared to phenazine methosulfate (PMS) with all of the listed organisms after 30 minutes. These results illustrate the improved sensitivity to most urinary tract infection microorganisms obtained with the present invention. It is also evident that the present invention shows an even response to most of the microorganisms. The only exception was the first *S. pyogenes* test which exhibited a low response with both ETAs tested because the microorganism was grown in BHI medium not supplemented with additional glucose. Excess glucose may be required for induction of metabolic enzymes.

Table VI

		ΔA 635 nm (30 minutes)	
<u>Microorganism</u>		<u>ETA III</u>	<u>PMS</u>
	<u><i>Pseudomonas aeruginosa</i></u> (ATCC 27853)	3.602	0.226
	<u><i>Escherchia coli</i></u> (ATCC 25922)	3.693	3.442
	<u><i>Staphylococcus aureus</i></u> (ATCC 25923)	3.448	0.956
	<u><i>Klebsiella pneumoniae</i></u> (ATCC 13883)	3.674	3.410
	<u><i>Streptococcus pyogenes</i></u> (ATCC 19615)	0.208	0.121
	<u><i>Streptococcus pyogenes</i>*</u> (ATCC 19615)	2.968	0.121
	<u><i>Streptococcus faecalis</i></u> (ATCC 19433)	3.478	3.312
	<u><i>Proteus vulgaris</i></u> (ATCC 13315)	3.669	3.378

* This culture of *S. pyogenes* was grown in glucose buffer medium.

Example 6 -

Determination of *E. coli* Using a Dry Analytical Element

A dry analytical element was prepared having the following format and components:

5 10 15 20	Spreading/ Reagent Layer	Poly(vinyltoluene-co-p-t-butyl- styrene-co-methacrylic acid) (61:37:2 weight ratio) beads (20-40 μ m diameter)	100-200 g/m ²
		Poly(n-butyl acrylate-co- styrene-co-2-acrylamido-2- methylpropane sulfonic acid) (70:20:10 weight ratio)	1-20 g/m ²
		RIND shown in Example 1	0.05-1 g/m ²
		D-glucose	0.05-5 g/m ²
		TRITON X-100 nonionic surfactant	0.05-5 g/m ²
		ETA III	0.01-10 g/m ²

Poly(ethylene terephthalate)
Support

A test solution was prepared with *E. coli* cells (5×10^8 cells/ml) in sodium phosphate buffer (0.05 molar, pH 7.5). A Control solution contained only buffer. An aliquot (10 μ l) of each solution was spotted on samples of the element. After 30 minutes of incubation at 37°C, the resulting reflection density (D_R) was measured in the element at 650 nm using a commercially available spectrophotometer. This procedure was done six times and the D_R results for each sample were averaged. The difference in D_R between the test solution average and the Control solution average was 0.094 indicating that the *E. coli* cells initiated reduction of the RIND compound.

Example 7 - Determination of *E. coli* Using RIND XX

In this example, assays of this invention using several of the ETAs of Table I above were compared to an assay using PMS. The reducible compound was the RIND compound shown in Example 2 above. The procedure followed was like that of Example 1. The release of dye was followed on a conventional spectrophotometer at 636 nm. Table VIII below lists the % dye released after 30 minutes as well as the % background at that time. All of the ETAs used generally promoted high dye release, but the assays of this invention had lower background.

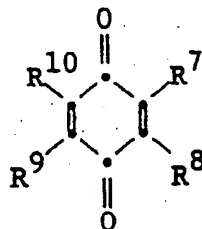
Table VIII

ETA	% Dye Release	% Background
PMS	100	4.5
I	100	2.7
II	100	1.3
III	100	1.8
IV	96	1.8
VIII	96	1.1
XVII	96	1.9
XXVI	100	3.1
XXVII	100	2.4
XXVIII	96	1.1

Claims

1. An aqueous composition for the determination of an analyte comprising:

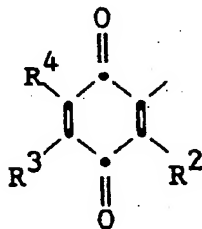
- (a) an electron transfer agent which is capable of being reduced by the analyte;
 - (b) a reducible compound which provides a detectable species when reduced by the reduced electron transfer agent; and
 - (c) a buffer which maintains the pH of the composition at 9 or less;
- the composition characterized wherein the electron transfer agent has the structure:



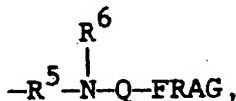
wherein R⁷ and R⁸ are independently hydrogen, alkyl having 1 to 10 carbon atoms, alkenyl having 1 to 10 carbon atoms, alkoxy having 1 to 10 carbon atoms, hydroxyalkyl having 1 to 10 carbon atoms, hydroxyalkoxy having 1 to 10 carbon atoms, alkoxyalkyl having 2 to 10 carbon atoms, alkoxyalkoxy having 2 to 10 carbon atoms, acetoxyalkyl having 1 to 10 carbon atoms in the alkyl portion, acetoxyalkoxy having 1 to 10 carbon atoms in the alkoxy portion, aryl having 6 to 12 carbon atoms, alkaryl having 7 to 10 carbon atoms, a heterocycle having 5 to 12 carbon, nitrogen, oxygen or sulfur atoms in the ring or a heteroalkyl having 5 to 12 carbon, nitrogen, oxygen or sulfur atoms in the ring, and

R⁹ and R¹⁰ are independently R⁷ or R⁸, or taken together supply the atoms to complete a 4- to 8-membered fused carbocyclic or heterocyclic ring with the quinone nucleus, provided at least one of R⁷, R⁸ and R⁹ and R¹⁰ is not hydrogen and the electron transfer agent has an E_{1/2} of from -320 to +400 mV as measured in an aqueous solution at pH 7, and

the reducible compound is a dichloroindophenol dye or a compound of the structure CAR-R¹ wherein CAR- is



R¹ is



R² and R⁴ are independently hydrogen, alkyl having 1 to 40 carbon atoms, aryl having 6 to 40 carbon atoms or an electron withdrawing group having a Hammett sigma value greater than 0.06,

R³ is R¹ hydrogen, alkyl having 1 to 40 carbon atoms, aryl having 6 to 40 atoms, or an electron withdrawing group having a Hammett sigma value greater than 0.06, or R³ and R⁴, taken together, represent the carbon atoms necessary to complete a fused carbocyclic ring with the quinone nucleus, the fused ring having 4 to 8 carbon atoms,

R⁵ is alkylene of 1 or 2 carbon atoms,

R⁶ is alkyl having 1 to 40 carbon atoms, cycloalkyl having 4 to 40 carbon atoms, heterocycle

having 5 to 40 carbon and heteroatoms or aryl having 6 to 40 carbon atoms, provided that when FRAG is a fluorogen, R⁶ is methyl,

Q is carbonyl or thiocarbonyl, and

FRAG is a shiftable detectable species which provides a detectable species when released from the reducible compound,

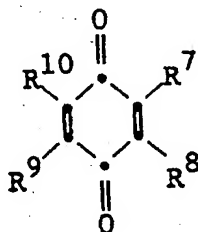
provided that when R¹ is replaced with H, CAR-H has an E_{1/2} of either from +100 to +400 mV when measured in water, or from -650 to -300 mV when measured in acetonitrile, and

further provided that when the reducible compound is reduced at pH 7, at least 50% of FRAG is released within 30 minutes.

2. A non-photosensitive, dry analytical element for the determination of an analyte comprising an absorbent carrier material,

the element characterized as containing:

- (a) an electron transfer agent which is capable of being reduced by an analyte and having the structure:

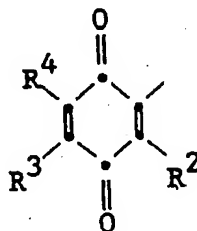


wherein R⁷ and R⁸ are independently hydrogen, alkyl having 1 to 10 carbon atoms, alkenyl having 1 to 10 carbon atoms, alkoxy having 1 to 10 carbon atoms, hydroxyalkyl having 1 to 10 carbon atoms, hydroxyalkoxy having 1 to 10 carbon atoms, alkoxyalkyl having 2 to 10 carbon atoms, alkoxyalkoxy having 2 to 10 carbon atoms, acetoxyalkyl having 1 to 10 carbon atoms in the alkyl portion, acetoxyalkoxy having 1 to 10 carbon atoms in the alkoxy portion, aryl having 6 to 12 carbon atoms, alkaryl having 7 to 10 carbon atoms, a heterocycle having 5 to 12 carbon, nitrogen, oxygen or sulfur atoms in the ring or a heteroalkyl having 5 to 12 carbon, nitrogen, oxygen or sulfur atoms in the ring, and

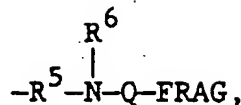
R⁹ and R¹⁰ are independently R⁷ or R⁸, or taken together supply the atoms to complete a 4- to 8-membered fused carbocyclic or heterocyclic ring with the quinone nucleus, provided at least one of R⁷, R⁸ and R⁹ and R¹⁰ is not hydrogen and the electron transfer agent has an E_{1/2} of from -320 to +400 mV as measured in an aqueous solution at pH 7;

- (b) a reducible compound which provides a detectable species when reduced by the reduced electron transfer agent,

the reducible compound being a dichloroindophenol dye or a compound of the structure CAR-R¹ wherein CAR- is



R¹ is



R² and R⁴ are independently hydrogen, alkyl having 1 to 40 carbon atoms, aryl having 6 to 40 carbon atoms or an electron withdrawing group having a Hammett sigma value greater than 0.06,

R³ is R¹, hydrogen, alkyl having 1 to 40 carbon atoms, aryl having 6 to 40 atoms, or an electron withdrawing group having a Hammett sigma value greater than 0.06, or R³ and R⁴, taken together, represent the carbon atoms necessary to complete a fused carbocyclic ring with the quinone nucleus, the fused ring having 4 to 8 carbon atoms,

R⁵ is alkylene of 1 or 2 carbon atoms,

R⁶ is alkyl having 1 to 40 carbon atoms, cycloalkyl having 4 to 40 carbon atoms, heterocycle having 5 to 40 carbon and heteroatoms or aryl having 6 to 40 carbon atoms, provided that when FRAG is a fluorogen, R⁶ is methyl,

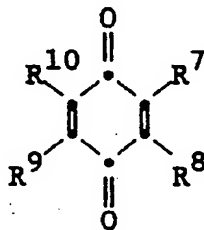
Q is carbonyl or thiocarbonyl, and

FRAG is a shiftable detectable species which provides a detectable species when released from the reducible compound,

provided that when R¹ is replaced with H, CAR-H has an E_{1/2} of either from +100 to +400 mV when measured in water, or from -650 to -300 mV when measured in acetonitrile, and

further provided that when the reducible compound is reduced at pH 7, at least 50% of FRAG is released within 30 minutes.

3. The element as claimed in claim 1 further comprising a non-photosensitive interactive composition for the analyte.
4. A method for the determination of an analyte in a liquid comprising the steps of:
 - A. at a pH of 9 or less, contacting a sample of the liquid with
 - (a) an electron transfer agent which is capable of being reduced by an analyte, and having the structure:

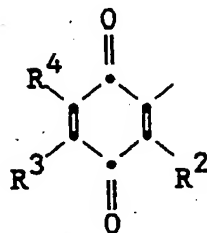


wherein R⁷ and R⁸ are independently hydrogen, alkyl having 1 to 10 carbon atoms, alkenyl having 1 to 10 carbon atoms, alkoxy having 1 to 10 carbon atoms, hydroxyalkyl having 1 to 10 carbon atoms, hydroxyalkoxy having 1 to 10 carbon atoms, alkoxyalkyl having 2 to 10 carbon atoms, alkoxyalkoxy having 2 to 10 carbon atoms, acetoxyalkyl having 1 to 10 carbon atoms in the alkyl portion, acetoxyalkoxy having 1 to 10 carbon atoms in the alkoxy portion, aryl having 6 to 12 carbon atoms, alkaryl having 7 to 10 carbon atoms, a heterocycle having 5 to 12 carbon, nitrogen, oxygen or sulfur atoms in the ring or a heteroalkyl having 5 to 12 carbon, nitrogen, oxygen or sulfur atoms in the ring, and

R⁹ and R¹⁰ are independently R⁷ or R⁸, or taken together supply the atoms to complete a 4- to 8-membered fused carbocyclic or heterocyclic ring with the quinone nucleus, provided at least one of R⁷, R⁸ and R⁹ and R¹⁰ is not hydrogen and the electron transfer agent has an E_{1/2} of from -320 to +400 mV as measured in an aqueous solution at pH 7;

(b) a reducible compound which provides a detectable species when reduced by the reduced electron transfer agent,

the reducible compound being a dichloroindophenol dye or a compound of the structure CAR-R¹ wherein CAR- is



R¹ is



R² and R⁴ are independently hydrogen, alkyl having 1 to 40 carbon atoms, aryl having 6 to 40 carbon atoms or an electron withdrawing group having a Hammett sigma value greater than 0.06,

R³ is R¹, hydrogen, alkyl having 1 to 40 carbon atoms, aryl having 6 to 40 atoms, or an electron withdrawing group having a Hammett sigma value greater than 0.06, or R³ and R⁴, taken together, represent the carbon atoms necessary to complete a fused carbocyclic ring with the quinone nucleus, the fused ring having 4 to 8 carbon atoms,

R⁵ is alkylene of 1 or 2 carbon atoms,

R⁶ is alkyl having 1 to 40 carbon atoms, cycloalkyl having 4 to 40 carbon atoms, heterocycle having 5 to 40 carbon and heteroatoms or aryl having 6 to 40 carbon atoms, provided that when FRAG is a fluorogen, R⁶ is methyl,

Q is carbonyl or thiocarbonyl; and

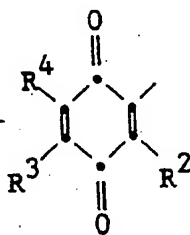
FRAG is a shiftable detectable species which provides a detectable species when released from the reducible compound,

provided that when R¹ is replaced with H, CAR-H has an E_{1/2} of either from +100 to +400 mV when measured in water, or from -650 to -300 mV when measured in acetonitrile, and

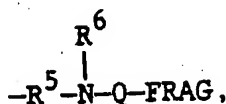
further provided that when the reducible compound is reduced at pH 7, at least 50% of FRAG is released within 30 minutes, and

B. detecting the detectable species in the liquid sample as an indication of the presence of the analyte.

5. The method as claimed in claim 4 for the determination of a non-living analyte in the presence of an interactive composition for the analyte.
6. The method as claimed in claim 4 for the determination of living cells.
7. The method as claimed in claim 6 for the determination of a microorganism.
8. The method as claimed in any of claims 4 to 7 wherein step B is carried out within 60 minutes of step A.
9. The invention as claimed in any of claims 1 to 8 wherein the reducible compound has the structure CAR-R¹ wherein CAR- is



R¹ is



R² and R⁴ are independently hydrogen, alkyl having 1 to 40 carbon atoms, aryl having 6 to 40 carbon atoms or an electron withdrawing group having a Hammett sigma value greater than 0.06,

R³ is R¹, hydrogen, alkyl having 1 to 40 carbon atoms, aryl having 6 to 40 atoms, or an electron withdrawing group having a Hammett sigma value greater than 0.06, or R³ and R⁴, taken together, represent the carbon atoms necessary to complete a fused carbocyclic ring with the quinone nucleus, the fused ring having 4 to 8 carbon atoms,

R⁵ is alkylene of 1 or 2 carbon atoms,

R⁶ is alkyl having 1 to 40 carbon atoms, cycloalkyl having 4 to 40 carbon atoms, heterocycle having 5 to 40 carbon and heteroatoms or aryl having 6 to 40 carbon atoms, provided that when FRAG is a fluorogen, R⁶ is methyl,

Q is carbonyl or thiocarbonyl, and

FRAG is a shiftable detectable species which provides a detectable species when released from the reducible compound,

provided that when R¹ is replaced with H, CAR-H has an E_{1/2} of either from +100 to +400 mV when measured in water, or from -650 to -300 mV when measured in acetonitrile, and

further provided that when the reducible compound is reduced at pH 7, at least 50% of FRAG is released within 30 minutes.

10. The invention as claimed in any of claims 1 to 9 wherein R⁷ and R⁸ are independently hydrogen, alkyl having 1 to 10 carbon atoms, alkoxy having 1 to 10 carbon atoms or hydroxyalkyl having 1 to 10 carbon atoms, and

R⁹ and R¹⁰ are independently R⁷ or R⁸, or taken together, supply the carbon atoms to complete a 6- to 8-membered fused carbocyclic ring with the quinone nucleus, and

the electron transfer agent has an E_{1/2} of from -185 to +400 mV as measured in aqueous solution at pH 7.

11. The invention as claimed in any of claims 1 to 10 wherein the electron transfer agent is 2,3,5-trimethyl-1,4-benzoquinone, 2,3-dimethyl-5-hydroxymethyl-1,4-benzoquinone or 2,3-dimethoxy-5-hydroxy-5-methyl-1,4-benzoquinone.

Revendications

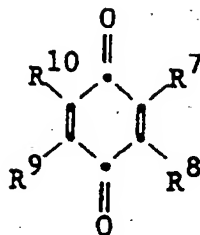
1. Composition aqueuse pour doser une substance à analyser comprenant :

a) un agent de transfert d'électrons qui peut être réduit par la substance à analyser ;

b) un composé réductible qui forme une espèce détectable lorsqu'il est réduit par l'agent de transfert d'électrons réduit ; et

c) un tampon qui maintient la composition à un pH inférieur ou égal à 9 ;

composition caractérisée en ce que l'agent de transfert d'électrons a la structure :

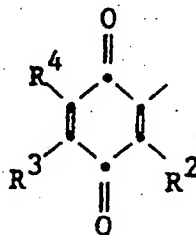


où

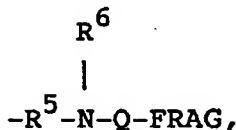
R⁷ et R⁸ sont chacun séparément un hydrogène, un groupe alkyle de 1 à 10 atomes de carbone, alkenyle de 1 à 10 atomes de carbone, alkoxy de 1 à 10 atomes de carbone, alkoxyaryle de 1 à 10 atomes de carbone, hydroxyalkoxy de 1 à 10 atomes de carbone, alkoxyalkyle de 2 à 10 atomes de carbone, alkoxyalkoxy de 2 à 10 atomes de carbone, acétoxyalkyle de 1 à 10 atomes de carbone dans la portion alkyle, acétoxyalkoxy de 1 à 10 atomes de carbone dans la portion alkoxy, aryle de 6 à 12 atomes de carbone, alkaryle de 7 à 10 atomes de carbone, un hétérocycle ayant de 5 à 12 atomes de carbone, d'azote, d'oxygène ou de soufre sur le cycle ou un groupe hétéroalkyle de 5 à 12 atomes de carbone, d'azote, d'oxygène ou de soufre sur le cycle, et

R⁹ et R¹⁰ sont chacun séparément R⁷ ou R⁸, ou bien ensemble sont les atomes nécessaires pour compléter avec le noyau quinone un cycle condensé carbocyclique ou hétérocyclique à 4 ou 8 chaînons, à condition qu'au moins l'un des radicaux R⁷, R⁸, et R⁹ et R¹⁰ ne soit pas un hydrogène et que l'agent de transfert d'électrons ait un E_{1/2} compris entre -320 et +400 mV mesuré en solution aqueuse à pH 7, et

le composé réductible est un colorant dichloroindophénol ou un composé de structure CAR-R¹ où CAR-est



R¹ est



R² et R⁴ sont chacun séparément un hydrogène, un radical alkyle de 1 à 40 atomes de carbone, aryle de 6 à 40 atomes de carbone ou un groupe accepteur d'électrons ayant une constante sigma de Hammett supérieure à 0,06,

R³ est R¹, un hydrogène, un radical alkyle de 1 à 40 atomes de carbone, aryle de 6 à 40 atomes de carbone ou un groupe accepteur d'électrons ayant une constante sigma de Hammett supérieure à 0,06, ou R³ et R⁴ ensemble représentent les atomes de carbone nécessaires pour compléter un noyau carbocyclique condensé avec le noyau quinone, le noyau condensé ayant 4 à 8 atomes de carbone,

R⁵ est un radical alkylène de 1 à 2 atomes de carbone,

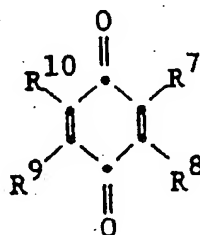
R⁶ est un radical alkyle de 1 à 40 atomes de carbone, cycloalkyle de 4 à 40 atomes de carbone, un hétérocycle de 5 à 40 atomes de carbone et hétéroatomes, ou aryle ayant de 6 à 40 atomes de carbone, avec la condition que quand FRAG est un fluorogène, R⁶ est méthyle,

Q est un radical carbonyle ou thiocarbonyle, et FRAG est une espèce détectable qui présente une

bande d'absorption décalée après avoir été libérée du composé réductible, avec la condition que lorsque R¹ est remplacé par H, CAR-H ait un E_{1/2} soit de +100 à +400 mV mesuré dans l'eau, soit de -650 à -300 mV mesuré dans l'acétonitrile, et avec la condition supplémentaire que le composé réductible soit réduit à pH 7, et qu'au moins 50% de FRAG soit libéré en 30 minutes.

2. Produit d'analyse à sec, non photosensible pour doser une substance à analyser comprenant un matériau porteur absorbant, élément caractérisé en ce qu'il contient :

a) un agent de transfert d'électrons qui peut être réduit par la substance à analyser et qui a la structure :

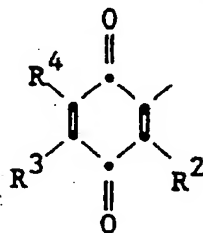


où

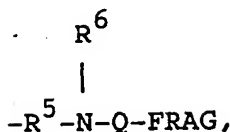
R⁷ et R⁸ sont chacun séparément un hydrogène, un groupe alkyle de 1 à 10 atomes de carbone, alkenyle de 1 à 10 atomes de carbone, alkoxy de 1 à 10 atomes de carbone, alkoxyaryle de 1 à 10 atomes de carbone, hydroxyalkoxy de 1 à 10 atomes de carbone, alkoxyalkyle de 2 à 10 atomes de carbone, alkoxyalkoxy de 2 à 10 atomes de carbone, acétoxyalkyle de 1 à 10 atomes de carbone dans la portion alkyle, acétoxyalkoxy de 1 à 10 atomes de carbone dans la portion alkoxy, aryle de 6 à 12 atomes de carbone, alkaryle de 7 à 10 atomes de carbone, un hétérocycle ayant de 5 à 12 atomes de carbone, d'azote, d'oxygène ou de soufre sur le cycle ou un groupe hétéroalkyle de 5 à 12 atomes de carbone, d'azote, d'oxygène ou de soufre sur le cycle, et

R⁹ et R¹⁰ sont chacun séparément R⁷ ou R⁸, ou bien ensemble sont les atomes nécessaires pour compléter avec le noyau quinone un cycle condensé carbocyclique ou hétérocyclique à 4 ou 8 chaînons à condition qu'au moins l'un des radicaux R⁷, R⁸, et R⁹ et R¹⁰ ne soit pas un hydrogène et que l'agent de transfert d'électrons ait un E_{1/2} compris entre -320 et +400 mV mesuré en solution aqueuse à pH 7, et

b) un composé réductible qui forme une espèce détectable lorsqu'il est réduit par l'agent de transfert d'électrons et qui est un colorant dichloroindophénol ou un composé de structure CAR-R¹ où CAR- est



R¹ est



R² et R⁴ sont chacun séparément

un hydrogène, un radical alkyle de 1 à 40 atomes de carbone, aryle de 6 à 40 atomes de carbone ou un groupe accepteur d'électrons ayant une constante sigma de Hammet supérieure à 0,06,

R³ est R¹, un hydrogène, un radical alkyle de 1 à 40 atomes de carbone, aryle de 6 à 40 atomes de carbone ou un groupe accepteur d'électrons ayant une constante sigma de Hammet supérieure à 0,06, ou R³ et R⁴ ensemble les atomes de carbone nécessaires pour compléter un noyau condensé ayant 4 à 8 atomes de carbone,

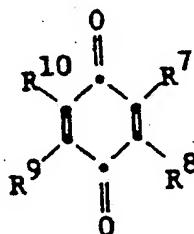
R₅ est un radical alkylène de 1 à 2 atomes de carbone,

R⁶ est un radical alkyle de 1 à 40 atomes de carbone, cycloalkyle de 4 à 40 atomes de carbone, un hétérocycle de 5 à 40 atomes de carbone et hétéroatomes, ou aryle ayant de 6 à 40 atomes de carbone, avec la condition que quand FRAG est un fluorogène, R⁵ est méthyle,

Q est un radical carbonyle ou thiocarbonyle, et FRAG est une espèce détectable qui présente une bande d'absorption décalée après avoir été libérée du composé réductible,

avec la condition que lorsque R¹ est remplacé par H, CAR-H ait un E_{1/2} soit de +100 à +400 mV mesuré dans l'eau, soit de -650 à -300 mV mesuré dans l'acétonitrile, et avec la condition supplémentaire que le composé réductible soit réduit à pH 7, et qu'au moins 50% de FRAG soit libéré en 30 minutes.

3. Produit selon la revendication 1 comprenant de plus une composition non photosensible qui réagit avec la substance à analyser.
4. Méthode de dosage d'une substance à analyser dans un liquide comprenant les étapes suivantes :
 - A. à un pH inférieur ou égal à 9, on met en contact un liquide avec
 - a) un agent de transfert d'électrons qui peut être réduit par la substance à analyser, et qui a la structure :

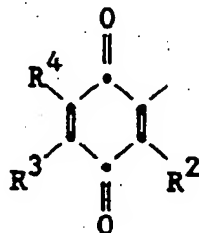


où

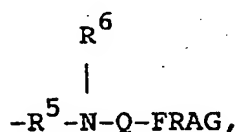
R⁷ et R⁸ sont chacun séparément un hydrogène, un groupe alkyle de 1 à 10 atomes de carbone, alkenyle de 1 à 10 atomes de carbone, alkoxy de 1 à 10 atomes de carbone, alkoxyaryle de 1 à 10 atomes de carbone, hydroxyalkoxy de 1 à 10 atomes de carbone, alkoxyalkyle de 2 à 10 atomes de carbone, alkoxyalkoxy de 2 à 10 atomes de carbone, acétoxyalkyle de 1 à 10 atomes de carbone dans la portion alkyle, acétoxyalkoxy de 1 à 10 atomes de carbone dans la portion alkoxy, aryle de 6 à 12 atomes de carbone, alkaryle de 7 à 10 atomes de carbone, un hétérocycle ayant de 5 à 12 atomes de carbone, d'azote, d'oxygène ou de soufre sur le cycle ou un groupe hétéroalkyle de 5 à 12 atomes de carbone, d'azote, d'oxygène ou de soufre sur le cycle, et

R⁹ et R¹⁰ sont chacun séparément R⁷ ou R⁸, ou bien ensemble sont les atomes nécessaires pour compléter avec le noyau quinone un cycle condensé carbocyclique ou hétérocyclique à 4 ou 8 chaînons à condition qu'au moins l'un des radicaux R⁷, R⁸, et R⁹ et R¹⁰ ne soit pas un hydrogène et que l'agent de transfert d'électrons ait un E_{1/2} compris entre -320 et +400 mV mesuré en solution aqueuse à pH 7, et

b) un composé réductible qui forme une espèce détectable lorsqu'il est réduit par l'agent de transfert d'électrons et qui est un colorant dichloroindophénol ou un composé de structure CAR-R¹ où CAR- est



R¹ est

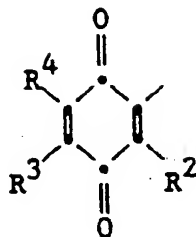


R² et R⁴ sont chacun séparément un hydrogène, un radical alkyle de 1 à 40 atomes de carbone, aryle de 6 à 40 atomes de carbone ou un groupe accepteur d'électrons ayant une constante sigma de Hammett supérieure à 0,06, R³ est R¹, un hydrogène, un radical alkyle de 1 à 40 atomes de carbone, aryle de 6 à 40 atomes de carbone ou un groupe accepteur d'électrons ayant une constante sigma de Hammett supérieure à 0,06, ou R³ et R⁴ ensemble représentent les atomes de carbone nécessaires pour compléter un noyau condensé ayant 4 à 8 atomes de carbone, R⁵ est un radical alkylène de 1 à 2 atomes de carbone, R⁶ est un radical alkyle de 1 à 40 atomes de carbone, cycloalkyle de 4 à 40 atomes de carbone, un hétérocycle de 5 à 40 atomes de carbone et hétéroatomes, ou aryle ayant de 6 à 40 atomes de carbone, avec la condition que quand FRAG est un fluorogène, R⁶ est méthyle, Q est un radical carbonyle ou thiocarbonyle, et FRAG est une espèce détectable qui présente une bande d'absorption décalée après avoir été libérée du composé réductible,

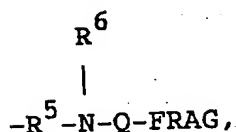
avec la condition que lorsque R¹ est remplacé par H, CAR-H ait un E_{1/2} soit de +100 à +400 mV mesuré dans l'eau, soit de -650 à -300 mV mesuré dans l'acétonitrile, et avec la condition supplémentaire que le composé réductible soit réduit à pH 7, et qu'au moins 50% de FRAG soit libéré en 30 minutes, et

B. on dose les espèces détectables dans l'échantillon liquide, qui indiquent la présence de la substance à analyser.

5. Méthode selon la revendication 4 pour doser une substance à analyser non-vivante en présence d'une composition qui réagit avec la substance à analyser.
6. Méthode selon la revendication 4 pour doser des cellules vivantes.
7. Méthode selon la revendication 6, pour doser des microorganismes.
8. Méthode selon l'une quelconque des revendications 4 à 7 dans laquelle l'étape B s'effectue moins de 60 minutes après l'étape A.
9. Invention selon l'une quelconque des revendications 1 à 8 dans laquelle le composé réductible a la structure CAR-R¹ où CAR- est



R¹ est



R² et R⁴ sont chacun séparément un hydrogène, un radical alkyle de 1 à 40 atomes de carbone, aryle de 6 à 40 atomes de carbone ou un groupe accepteur d'électrons ayant une constante sigma de Hammett supérieure à 0,06, R³ est R¹, un hydrogène, un radical alkyle de 1 à 40 atomes de carbone, aryle de 6 à 40 atomes de carbone ou un groupe accepteur d'électrons ayant une constante sigma de Hammett supérieure à 0,06, ou R³ et R⁴ ensemble représentent les atomes de carbone nécessaires pour compléter un noyau condensé ayant 4 à 8 atomes de carbone, R⁵ est un radical alkylène de 1 à 2 atomes de carbone, R⁶ est un radical alkyle de 1 à 40 atomes de carbone, cycloalkyle de 4 à 40 atomes de carbone, un hétérocycle de 5 à 40 atomes de carbone et hétéroatomes, ou aryle ayant de 6 à 40 atomes de carbone, avec la condition que quand FRAG est un fluorogène, R⁶ est méthyle, Q est un radical carbonyle ou thiocarbonyle, et FRAG est une espèce détectable qui présente une bande d'absorption décalée après avoir été libérée du composé réductible, avec la condition que lorsque R¹ est remplacé par H, CAR-H ait un E_{1/2} soit de +100 à +400 mV mesuré dans l'eau, soit de -650 à -300 mV mesuré dans l'acétonitrile, et avec la condition supplémentaire que le composé réductible soit réduit à pH 7, et qu'au moins 50% de FRAG soit libéré en 30 minutes.

10. Invention selon l'une quelconque des revendications 1 à 9 où R⁷ et R⁸ sont chacun séparément un hydrogène, un groupe alkyle de 1 à 10 atomes de carbone, alkoxy de 1 à 10 atomes de carbone ou hydroxyalkyle de 1 à 10 atomes de carbone, et R⁹ et R¹⁰ sont chacun séparément R⁷ ou R⁸, ou bien ensemble sont les atomes nécessaires pour compléter avec le noyau quinone un cycle condensé carbocyclique ou hétérocyclique à 6 ou 8 chaînons, et l'agent de transfert d'électrons a un E_{1/2} compris entre -185 et +400 mV mesuré en solution aqueuse à pH 7.

11. Invention selon l'une quelconque des revendications 1 à 10 dans laquelle l'agent de transfert d'électrons est la 2,3,5-triméthyl-1,4-benzoquinone, la 2,3-diméthyl-5-hydroxyméthyl-1,4-benzoquinone ou la 2,3-diméthoxy-5-hydroxy-5-méthyl-1,4-benzoquinone.

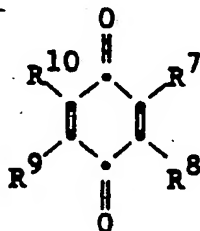
Patentansprüche

1. Wäßrige Zusammensetzung für die Bestimmung eines Analyten mit:

- einem Elektronenübertragungsmittel, das durch den Analyten reduziert werden kann;
- einer reduzierbaren Verbindung, die einen nachweisbaren Stoff zu erzeugen vermag, wenn sie durch das Elektronenübertragungsmittel reduziert wird und

(c) einem Puffer, der den pH-Wert der Zusammensetzung bei 9 oder darunter hält,

dadurch gekennzeichnet, daß das Elektronenübertragungsmittel der folgenden Struktur entspricht:

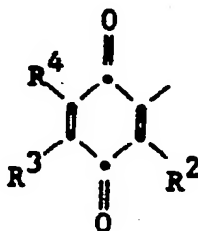


in der bedeuten:

R und R⁸ unabhängig voneinander Wasserstoff, Alkyl mit 1 bis 10 Kohlenstoffatomen, Alkenyl mit 1 bis 10 Kohlenstoffatomen, Alkoxy mit 1 bis 10 Kohlenstoffatomen, Hydroxyalkyl mit 1 bis 10 Kohlenstoffatomen, Hydroxyalkoxy mit 1 bis 10 Kohlenstoffatomen, Alkoxyalkyl mit 2 bis 10 Kohlenstoffatomen, Alkoxyalkoxy mit 2 bis 10 Kohlenstoffatomen, Acetoxyalkyl mit 1 bis 10 Kohlenstoffatomen im Alkylrest, Acetoxyalkoxy mit 1 bis 10 Kohlenstoffatomen im Alkoxyrest, Aryl mit 6 bis 12 Kohlenstoffatomen, Alkaryl mit 7 bis 10 Kohlenstoffatomen, einen Heterocyclus mit 5 bis 12 Kohlenstoff-, Stickstoff-, Sauerstoff- oder Schwefelatomen im Ring oder einen Alkylheterocyclus mit 5 bis 12 Kohlenstoff-, Stickstoff-, Sauerstoff- oder Schwefelatomen im Ring, und

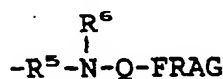
R⁹ und R¹⁰ unabhängig voneinander gleich R⁷ oder R⁸ oder gemeinsam die Atome, die zur Vervollständigung eines 4- bis 8-gliedrigen, an den Chinonring ankondensierten carbocyclischen oder heterocyclischen Ringes erforderlich sind, wobei gilt, daß mindestens einer der Substituenten R⁷, R⁸ und R⁹ und R¹⁰ nicht Wasserstoff ist und daß das Elektronenübertragungsmittel einen E_{1/2}-Wert von -320 bis +400mV, gemessen in einer wäßrigen Lösung bei einem pH-Wert von 7 aufweist, und daß

die reduzierbare Verbindung ein Dichloroindolphenol-Farbstoff ist oder eine Verbindung der Struktur CAR-R¹, in der CAR- für einen Rest der Formel:



steht,

R¹ gleich



ist,

R² und R⁴ unabhängig voneinander Wasserstoff, Alkyl mit 1 bis 40 Kohlenstoffatomen, Aryl mit 6 bis 40 Kohlenstoffatomen oder eine elektronenabziehende Gruppe mit einem Hammett-Sigma-Wert von größer als 0,06 bedeuten,

R³ gleich R¹, Wasserstoff, Alkyl mit 1 bis 40 Kohlenstoffatomen, Aryl mit 6 bis 40 Kohlenstoffatomen, oder eine elektronenabziehende Gruppe mit einem Hammett-Sigma-Wert von größer als 0,06 ist, oder R³ und R⁴ gemeinsam für die Kohlenstoffatome stehen, die zur Vervollständigung eines an den Chinonkern ankondensierten carbocyclischen Ringes mit 4 bis 8 Kohlenstoffatomen erforderlich sind,

R⁵ gleich Alkyl mit 1 oder 2 Kohlenstoffatomen ist;

R⁶ gleich Alkyl mit 1 bis 40 Kohlenstoffatomen, Cycloalkyl mit 4 bis 40 Kohlenstoffatomen, Heterocycl mit 5 bis 40 Kohlenstoff- und Heteroatomen oder Aryl mit 6 bis 40 Kohlenstoffatomen ist, wobei gilt, daß wenn FRAG ein Fluorogen ist, R⁶ die Bedeutung von Methyl hat,

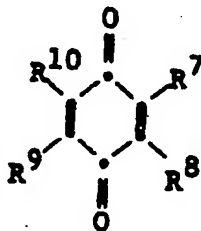
Q gleich Carbonyl oder Thiocarbonyl ist, und

FRAG ein verschiebbarer nachweisbarer Rest ist, der einen nachweisbaren Stoff liefert, wenn er von der reduzierbaren Verbindung freigesetzt wird,

wobei gilt, daß wenn R¹ durch H ersetzt wird, CAR-H einen E_{1/2}-Wert von entweder +100 bis +400 mV, gemessen in Wasser oder von -650 bis -300 mV, gemessen in Acetonitril hat und wobei ferner gilt, daß, wenn die reduzierbare Verbindung bei einem pH-Wert von 7 reduziert wird, mindestens 50 % von FRAG innerhalb von 30 Minuten freigesetzt werden.

2. Nicht-photosensitives, trockenes analytisches Element für den Nachweis eines Analyten mit einem absorbierenden Trägermaterial, dadurch gekennzeichnet, daß es enthält:

(a) ein Elektronenübertragungsmittel, das durch den Analyten reduziert werden kann und der folgenden Struktur entspricht:



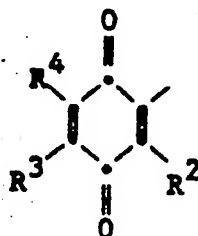
in der bedeuten:

R⁷ und R⁸ unabhängig voneinander Wasserstoff, Alkyl mit 1 bis 10 Kohlenstoffatomen, Alkenyl mit 1 bis 10 Kohlenstoffatomen, Alkoxy mit 1 bis 10 Kohlenstoffatomen, Hydroxyalkyl mit 1 bis 10 Kohlenstoffatomen, Hydroxyalkoxy mit 1 bis 10 Kohlenstoffatomen, Alkoxyalkyl mit 2 bis 10 Kohlenstoffatomen, Alkoxyalkoxy mit 2 bis 10 Kohlenstoffatomen, Acetoxyalkyl mit 1 bis 10 Kohlenstoffatomen im Alkylrest, Acetoxyalkoxy mit 1 bis 10 Kohlenstoffatomen im Alkoxyrest, Aryl mit 6 bis 12 Kohlenstoffatomen, Alkaryl mit 7 bis 10 Kohlenstoffatomen, einen Heterocycl mit 5 bis 12 Kohlenstoff-, Stickstoff-, Sauerstoff- oder Schwefelatomen im Ring oder einen Alkylheterocycl mit 5 bis 12 Kohlenstoff-, Stickstoff-, Sauerstoff- oder Schwefelatomen im Ring, und

R⁹ und R¹⁰ unabhängig voneinander gleich R⁷ oder R⁸ oder gemeinsam die Atome, die zur Vervollständigung eines 4- bis 8-gliedrigen, an den Chinonring ankondensierten carbocyclischen oder heterocyclischen Ringes erforderlich sind, wobei gilt, daß mindestens einer der Substituenten R⁷, R⁸ und R⁹ und R¹⁰ nicht Wasserstoff ist und daß das Elektronenübertragungsmittel einen E_{1/2}-Wert von -320 bis +400mV, gemessen in einer wäßrigen Lösung bei einem pH-Wert von 7 aufweist, und daß

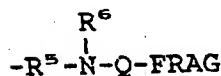
(b) eine reduzierbare Verbindung, die einen nachweisbaren Stoff zu erzeugen vermag, wenn sie durch das Elektronenübertragungsmittel reduziert wird, wobei

die reduzierbare Verbindung ein Dichloroindolphenol-Farbstoff ist oder eine Verbindung der Struktur CAR-R¹, in der CAR- für einen Rest der Formel:



steht,

R¹ gleich



ist,

R² und R⁴ unabhängig voneinander Wasserstoff, Alkyl mit 1 bis 40 Kohlenstoffatomen, Aryl mit 6 bis 40 Kohlenstoffatomen oder eine elektronenabziehende Gruppe mit einem Hammett-Sigma-Wert von größer als 0,06 bedeuten,

R³ gleich R¹, Wasserstoff, Alkyl mit 1 bis 40 Kohlenstoffatomen, Aryl mit 6 bis 40 Kohlenstoffatomen, oder eine elektronenabziehende Gruppe mit einem Hammett-Sigma-Wert von größer als 0,06 ist, oder R³ und R⁴ gemeinsam für die Kohlenstoffatome stehen, die zur Vervollständigung eines an den Chinonkern ankondensierten carbocyclischen Ringes mit 4 bis 8 Kohlenstoffatomen erforderlich sind,

R⁵ gleich Alkylen mit 1 oder 2 Kohlenstoffatomen ist;

R⁶ gleich Alkyl mit 1 bis 40 Kohlenstoffatomen, Cycloalkyl mit 4 bis 40 Kohlenstoffatomen, Heterocyclyl mit 5 bis 40 Kohlenstoff- und Heteroatomen oder Aryl mit 6 bis 40 Kohlenstoffatomen ist, wobei gilt, daß wenn FRAG ein Fluorogen ist, R⁶ die Bedeutung von Methyl hat,

Q gleich Carbonyl oder Thiocarbonyl ist, und

FRAG ein verschiebbarer nachweisbarer Rest ist, der einen nachweisbaren Stoff liefert, wenn er von der reduzierbaren Verbindung freigesetzt wird,

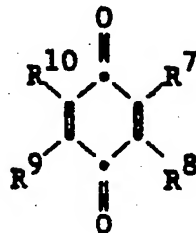
wobei gilt, daß, wenn R¹ durch H ersetzt wird, CAR-H einen E_{1/2}-Wert von entweder +100 bis +400 mV, gemessen in Wasser oder von -650 bis -300 mV, gemessen in Acetonitril hat und wobei ferner gilt, daß, wenn die reduzierbare Verbindung bei einem pH-Wert von 7 reduziert wird, mindestens 50 % von FRAG innerhalb von 30 Minuten freigesetzt werden.

3. Element nach Anspruch 2, dadurch gekennzeichnet, daß es ferner eine nicht-photosensitive reaktionsfähige Zusammensetzung für den Analyten enthält.

4. Verfahren zur Bestimmung eines Analyten in einer Flüssigkeit mit den Stufen:

A. Inkontaktbringen einer Probe der Flüssigkeit bei einem pH-Wert von 9 oder weniger mit

(a) einem Elektronenübertragungsmittel, das durch einen Analyten reduziert werden kann und der folgenden Struktur entspricht:



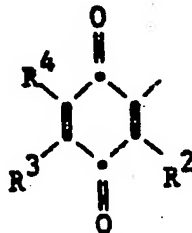
in der bedeuten:

R⁷ und R⁸ unabhängig voneinander Wasserstoff, Alkyl mit 1 bis 10 Kohlenstoffatomen, Alkenyl mit 1 bis 10 Kohlenstoffatomen, Hydroxyalkyl mit 1 bis 10 Kohlenstoffatomen, Hydroxyalkoxy mit 1 bis 10 Kohlenstoffatomen, Alkoxyalkyl mit 2 bis 10 Kohlenstoffatomen, Alkoxyalkoxy mit 2 bis 10 Kohlenstoffatomen, Acetoxyalkyl mit 1 bis 10 Kohlenstoffatomen im Alkylrest, Acetoxyalkoxy mit 1 bis 10 Kohlenstoffatomen im Alkoxyrest, Aryl mit 6 bis 12 Kohlenstoffatomen, Alkaryl mit 7 bis 10 Kohlenstoffatomen, einen Heterocyclus mit 5 bis 12 Kohlenstoff-, Stickstoff-, Sauerstoff- oder Schwefelatomen im Ring oder einen Alkylheterocyclus mit 5 bis 12 Kohlenstoff-, Stickstoff-, Sauerstoff- oder Schwefelatomen im Ring und

R⁹ und R¹⁰ unabhängig voneinander gleich R⁷ oder R⁸ oder gemeinsam die Atome, die zur Vervollständigung eines 4- bis 8-gliedrigen, an den Chinonring ankondensierten carbocyclischen oder heterocyclischen Ringes erforderlich sind, wobei gilt, daß mindestens einer der Substituenten R⁷, R⁸ und R⁹ und R¹⁰ nicht Wasserstoff ist und daß das Elektronenübertragungsmittel einen E_{1/2}-Wert von -320 bis +400mV, gemessen in einer wäßrigen Lösung bei einem pH-Wert von 7 aufweist,

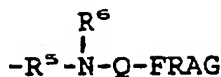
(b) einer reduzierbaren Verbindung, die einen nachweisbaren Stoff liefert, wenn sie durch das reduzierte Elektronenübertragungsmittel reduziert wird, wobei

die reduzierbare Verbindung ein Dichloroindolphenol-Farbstoff ist oder eine Verbindung der Struktur CAR-R¹, in der CAR- für einen Rest der Formel:



steht,

R¹ gleich



ist,

R² und R⁴ unabhängig voneinander Wasserstoff, Alkyl mit 1 bis 40 Kohlenstoffatomen, Aryl mit 6 bis 40 Kohlenstoffatomen oder eine elektronenabziehende Gruppe mit einem Hammett-Sigma-Wert von größer als 0,06 bedeuten,

R³ gleich R¹, Wasserstoff, Alkyl mit 1 bis 40 Kohlenstoffatomen, Aryl mit 6 bis 40 Kohlenstoffatomen, oder eine elektronenabziehende Gruppe mit einem Hammett-Sigma-Wert von größer als 0,06 ist, oder R³ und R⁴ gemeinsam für die Kohlenstoffatome stehen, die zur Vervollständigung eines an den Chinonkern ankondensierten carbocyclischen Ringes mit 4 bis 8 Kohlenstoffatomen erforderlich sind,

R⁵ gleich Alkylen mit 1 oder 2 Kohlenstoffatomen ist;

R⁶ gleich Alkyl mit 1 bis 40 Kohlenstoffatomen, Cycloalkyl mit 4 bis 40 Kohlenstoffatomen, Heterocyclyl mit 5 bis 40 Kohlenstoff- und Heteroatomen oder Aryl mit 6 bis 40 Kohlenstoffatomen ist, wobei gilt, daß wenn FRAG ein Fluorogen ist, R⁶ die Bedeutung von Methyl hat,

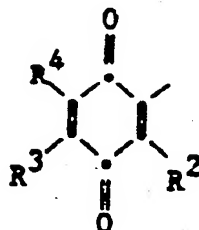
Q gleich Carbonyl oder Thiocarbonyl ist, und

FRAG ein verschiebbarer nachweisbarer Rest ist, der einen nachweisbaren Stoff liefert, wenn er von der reduzierbaren Verbindung freigesetzt wird,

wobei gilt, daß, wenn R¹ durch H ersetzt wird, CAR-H einen E_{1/2}-Wert von entweder +100 bis +400 mV, gemessen in Wasser oder von -650 bis -300 mV, gemessen in Acetonitril hat und wobei ferner gilt, daß, wenn die reduzierbare Verbindung bei einem pH-Wert von 7 reduziert wird, mindestens 50 % von FRAG innerhalb von 30 Minuten freigesetzt werden, und

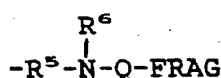
B. Nachweis des nachweisbaren Stoffes in der Flüssigkeitsprobe als Anzeichen für das Vorhandensein des Analyten.

5. Verfahren nach Anspruch 4 zur Bestimmung eines nicht-lebenden Analyten in Gegenwart einer reaktiven Zusammensetzung für den Analyten.
6. Verfahren nach Anspruch 4 zur Bestimmung lebender Zellen.
7. Verfahren nach Anspruch 6 zur Bestimmung eines Mikroorganismus.
8. Verfahren nach einem der Ansprüche 4 bis 7, in dem die Stufe B innerhalb von 60 Minuten nach der Stufe A durchgeführt wird.
9. Die Erfindung nach einem der Ansprüche 1 bis 8, in der die reduzierbare Verbindung der Struktur CAR-R¹ entspricht; in der CAR- für einen Rest der Formel:



steht,

R¹ gleich



ist,

R² und R⁴ unabhängig voneinander Wasserstoff, Alkyl mit 1 bis 40 Kohlenstoffatomen, Aryl mit 6 bis 40 Kohlenstoffatomen oder eine elektronenabziehende Gruppe mit einem Hammett-Sigma-Wert von größer als 0,06 bedeuten,

R³ gleich R¹, Wasserstoff, Alkyl mit 1 bis 40 Kohlenstoffatomen, Aryl mit 6 bis 40 Kohlenstoffatomen, oder eine elektronenabziehende Gruppe mit einem Hammett-Sigma-Wert von größer als 0,06 ist, oder R³ und R⁴ gemeinsam für die Kohlenstoffatome stehen, die zur Vervollständigung eines an den Chinonkern ankondensierten carbocyclischen Ringes mit 4 bis 8 Kohlenstoffatomen erforderlich sind,

R⁵ gleich Alkyl mit 1 oder 2 Kohlenstoffatomen ist,

R⁶ gleich Alkyl mit 1 bis 40 Kohlenstoffatomen, Cycloalkyl mit 4 bis 40 Kohlenstoffatomen, Heterocyclyl mit 5 bis 40 Kohlenstoff- und Heteroatomen oder Aryl mit 6 bis 40 Kohlenstoffatomen ist, wobei gilt, daß wenn FRAG ein Fluorogen ist, R⁶ die Bedeutung von Methyl hat,

Q gleich Carbonyl oder Thiocarbonyl ist, und

FRAG ein verschiebbarer nachweisbarer Rest ist, der einen nachweisbaren Stoff liefert, wenn er von der reduzierbaren Verbindung freigesetzt wird,

wobei gilt, daß, wenn R¹ durch H ersetzt wird, CAR-H einen E_{1/2}-Wert von entweder +100 bis +400 mV, gemessen in Wasser oder von -650 bis -300 mV, gemessen in Acetonitril hat und wobei ferner gilt, daß, wenn die reduzierbare Verbindung bei einem pH-Wert von 7 reduziert wird, mindestens 50 % von FRAG innerhalb von 30 Minuten freigesetzt werden.

10. Die Erfindung nach einem der Ansprüche 1 bis 9, in der R⁷ und R⁸ unabhängig voneinander stehen für Wasserstoff, Alkyl mit 1 bis 10 Kohlenstoffatomen, Alkoxy mit 1 bis 10 Kohlenstoffatomen oder Hydroxyalkyl mit 1 bis 10 Kohlenstoffatomen und

R⁹ und R¹⁰ unabhängig voneinander die Bedeutung von R⁷ oder R⁸ haben, oder gemeinsam für die Kohlenstoffatome stehen, die zur Vervollständigung eines 6- oder 8-gliedrigen, an den Chinonkern ankondensierten carbocyclischen Ringes stehen und

in der das Elektronenübertragungsmittel einen E_{1/2}-Wert von -185 bis +400 mV, gemessen in wäßriger Lösung bei einem pH-Wert von 7 hat.

11. Die Erfindung nach einem der Ansprüche 1 bis 10, in der das Elektronenübertragungsmittel 2,3,5-

Trimethyl-1,4-benzochinon; 2,3-Dimethyl-5-hydroxymethyl-1,4-benzochinon oder 2,3-Dimethoxy-5-hydroxy-5-methyl-1,4-benzochinon ist.

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